PB81-153843

Assessment of Mutagenic Potential of Mixtures of Organic Substances in Renovated Water

Syracuse Research Corp., New York

Prepared for

Health Effects Research Lab.
Cincinnati, OH

Feb 81

U.S. Department of Commerce
National Technical Information Service
NTIS.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/1-81-016		PB 81-153843

4. TITLE AND SUBTITLE	5. REPORT DATE
Assessment of Mutagenic Potential of Mixtures of Organic Substances in Renovated Water	FEBRUARY 1981 ISSUING DATE 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) M.W. Neal, L. Mason, D.J. Schwartz, J. Saxena	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Syracuse Research Corporation Merrill Lane Syracuse, New York 13210	10. PROGRAM ELEMENT NO. C27B1A 11. CONTRACT/GRANT NO. R804813
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research & Development U.S. Environmental Protection Agency Cincinnati, Ohio 45268	13. TYPE OF REPORT AND PERIOD COVERED Final, 1/1/77 to 6/16/80 14. SPONSORING AGENCY CODE EPA/600/10
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

An investigation was undertaken to evaluate the performance of several advanced wastewater treatment plants having biological, physical chemical, or soils treatment systems for removing or introducing mutagenic substances. Samples were taken before and after treatment and sometimes at intermediate points. Both concentrated and unconcentrated wastewaters were used. Three different concentration methods were utilized and compared. Salmonella typhimurium, Saccharomyces cerevisiae, and mammalian cells were used in the test systems.

Mutagenic activity was found to pass through most treatment plants and sometimes increased during treatment. Wastewater with no industrial wastes and strictly of domestic origin did not contain mutagenic properties.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Water treatment, waste water mutagens, organic compounds, bioassay, concentrates, toxicity	Ames test	68D 68G

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to the Public	Unclassified	
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

PB81-153842

EPA 600/1-81-016
February 1980

ASSESSMENT OF MUTAGENIC POTENTIAL OF MIXTURES OF ORGANIC SUBSTANCES IN RENOVATED WATER

by

Michael W. Neal
Lawrence Mason, Jr.
David J. Schwartz
Jeet Saxena

Syracuse Research Corporation
Life and Material Sciences Division
Syracuse, New York 13210

Grant No. R-804813

Project Officer

Herbert R. Pahren
Epidemiology Division
Health Effects Research Laboratory
Cincinnati, Ohio 45268

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

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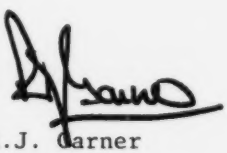
FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and governmental concern about the dangers of pollution to the health and welfare to the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our national environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to the valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

This report describes the performance of selected advanced wastewater treatment plants in removing mutagenic activity from the wastewater. Where possible the activity was related to organic fractions. With a knowledge of such process performance, we can obtain a better understanding of the factors necessary to improve effluent quality.



R.J. Garner
Director
Health Effects Research Laboratory

ABSTRACT

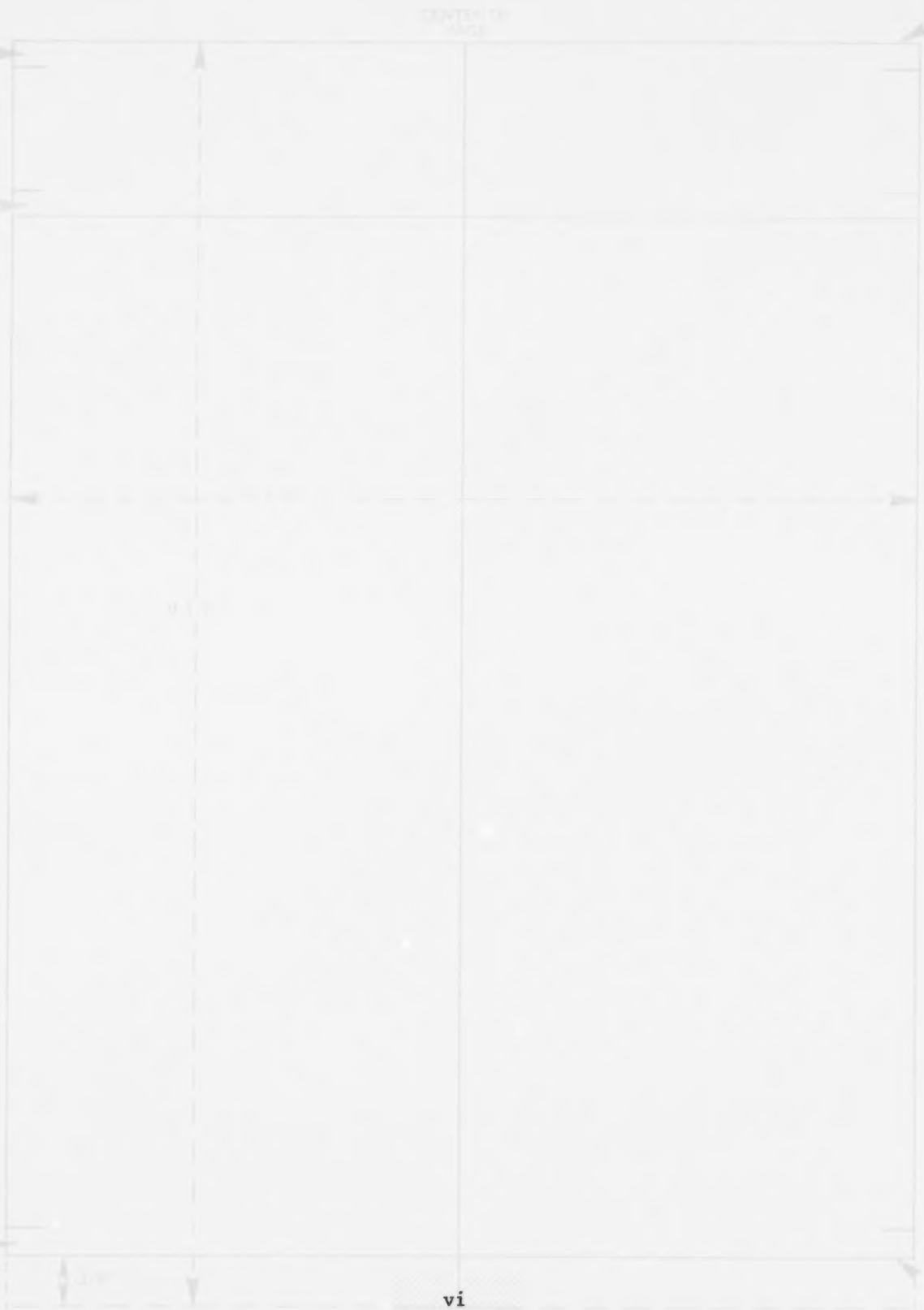
The investigation was undertaken to evaluate the performance of selected advanced wastewater treatment plants for removing (or introducing) mutagenic chemicals, and to determine the distribution of the detected activity among various classes of chemical compounds. The study utilized specially constructed strains of Salmonella typhimurium and Saccharomyces cerevisiae to assess mutagenic activity and mammalian cells, BHK 21 Cℓ13, to a limited extent, to determine transforming activity. Unconcentrated wastewaters were assayed by making media from filter sterilized test water. Crude organic mixtures and separated fractions of wastewater were evaluated using liquid suspension and/or plate incorporation assay. In an effort to recover a wide variety of organic contaminants present in wastewater/renovated wastewater, three independent concentration methods were utilized. These included sorption on polyurethane foam plugs, sorption on XAD resin, and solvent extraction.

The final effluent from Piscataway AWT process (physical-chemical) was found to contain mutagens belonging to the chemical classes of weak acid, basic, aromatic neutral, aliphatic neutral compounds, and many highly polar compounds which could not be recovered by any of the concentration methods employed. Effluent from Bay Park AWT process which utilizes a combination of biological and physical-chemical treatment methods, was shown to contain activity in the weak acid, ether insoluble and water soluble classes. It appeared that some of the activity detected in the renovated wastewaters may have been introduced during the renovation process. Alternatively, the renovation process, by removing toxicants and/or masking agents, may have made it possible to detect mutagenic compounds originally present in the influent. The efficiency of the rapid sand filtration process at Lake George could not be assessed because of the absence of active compounds in the wastewaters subjected to sand filtration. Wastewaters strictly of domestic origin were free of mutagenic activity. The data showed that prolonged contact with natural delta sand beds did not induce microbial transformation of mutagen precursors to mutagens. Studies involving dosing of simulated sand columns with wastewaters, in which the presence of mutagens had been established, showed that the treatment was effectively able to remove amphoteric, weak acid, and basic classes of mutagens, but not ether insoluble, water soluble, and strong acid classes.

The combined activity of all the separated fractions was generally found to be higher than that of the crude concentrates, possibly indicating the presence of toxicants and/or masking agents in the concentrates. Among the concentration methods used, polyurethane foam plugs showed relatively better performance towards collection of mutagenic compounds from wastewaters/renovated wastewaters. Polyurethane foam plugs also generally collected fewer toxicants. Mutagenic response by the wastewaters was observed mostly with

the Ames' Salmonella assay. The yeast assay lacked the required sensitivity for detection of mutagens in wastewater. The BHK cell transformation system proved unsuitable in this study because of its excessive time requirement and erratic behavior.

This report was submitted by Syracuse Research Corporation in fulfillment of Grant No. R 804813 from the U.S. Environmental Protection Agency and Grant-in-Aid from the American Petroleum Institute. This report covers a period from 1-1-1977 to 6-16-1980, and work was completed on 12-30-79.



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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AWT	-- Advanced wastewater treatment
BHK	-- Baby hamster kidney
DMSO	-- Dimethylsulfoxide
LGVSTP	-- Lake George Village Sewage Treatment Plant
µg	-- microgram
PAH	-- Polynuclear aromatic hydrocarbon
TOC	-- Total organic compound
S-9 Mix	-- Rat liver homogenate plus cofactors for metabolic activation
YEPD	-- Yeast extract peptone dextrose broth

ACKNOWLEDGEMENTS

Syracuse Research Corporation wishes to acknowledge the assistance of the staff of Piscataway Model AWT Plant, Piscataway, MD; Niagara Falls Municipal Wastewater Treatment Plant, Niagara Falls, NY; and Bay Park Wastewater Renovation Plant, E. Rockaway, NY, for their assistance and cooperation in sampling. The assistance of Dr. Donald Aulenbach of the Rensselaer Polytechnic Institute in obtaining the effluent samples from the rapid sand filtration process, and in performing the study with simulated sand columns, is also gratefully acknowledged.

Cost sharing by the American Petroleum Institute at the level of 5% is appreciated. API project officers were Dr. Neill K. Weaver and Dr. Eric Cowell.

SECTION 1

INTRODUCTION

As clean water resources become scarce and the need to protect our environment becomes urgent, wastewater reclamation will be increasingly practiced. Current reuse applications of wastewater are limited to non-potable purposes; e.g., irrigation, recreation, and industry. However, in certain geographical locations, water shortages are so acute that supplementing potable water supplies with reclaimed wastewater is being considered. While indirect reuse of wastewater from surface waters is commonplace, it is important to investigate fully the potential hazards from the direct consumption of renovated wastewaters before permitting potable reuse.

The health problems associated with the use of wastewater are two-fold: (a) microbiological health risks (e.g., presence of infectious virus) and (b) health effects of toxic chemicals. The toxic substances in reclaimed wastewater could be organics and/or heavy metals. The uncontrolled increase in the use of new, stable, and often complex synthetic compounds in industry and agriculture has led to concern over the appearance of these compounds in reclaimed wastewater. Organics in wastewater are of special concern, because wastewaters contain an organic mixture of unknown composition rather than single discrete compounds. The advanced wastewater renovation technology available today leaves some potentially hazardous chemicals unchanged. The residual concentration of organics in wastewaters renovated with the current technology has been reported to be of the order of 3 to 10 ppm (Shuval and Gruener, 1973; Warner and English, 1978). Also, some hazardous chemicals are introduced during the treatment itself; for example, formation of chlorinated hydrocarbons during chlorination of wastewaters (Glaze and Henderson, 1975). The problem is further magnified, because of the fact that residual trace pollutants in renovated wastewater vary widely from one situation to the next, depending on the nature of the individual waste which enters the sewage system. For example, petroleum products and refinery waste introduce various polynuclear aromatic hydrocarbons which are among the possible mutagenic and carcinogenic substances to appear in municipal wastewater.

Exposure to chemicals through consumption of reclaimed wastewater is expected to be at low levels but of long-term (often lifetime) duration. These conditions are ideally suited for chemically- induced carcinogenic and genotoxic effects. The grave and irreversible nature of the diseases renders the presence of mutagenic/carcinogenic chemicals in reclaimed wastewater to be of special concern. Furthermore, there is a general agreement that a completely safe exposure level of carcinogenic/mutagenic chemicals does not exist.

The present investigation was undertaken to examine the presence of mutagenic activity in municipal wastewater, with special emphasis on organic chemicals, and to evaluate the performance of available advanced wastewater treatment methods to remove such activity. Further, the distribution of the described activity among various classes of chemical compounds was studied in an attempt to identify the active fractions. The study was conducted utilizing rapid in vitro bioassays for detection of mutagenic/carcinogenic activity and, therefore, was expected to provide a preliminary assessment regarding the mutagenic/carcinogenic hazards linked to the potential potable use of reclaimed wastewater. The results of this investigation should be of value to the Environmental Protection Agency and others in identifying the problem areas and in setting priorities for in-depth and more specialized toxicological studies. Knowing that certain wastewaters have toxic properties, treatment systems may be designed to remove the toxic components or sources of such components, for example certain industries could provide pretreatment measures.

SECTION 2

CONCLUSIONS

Low levels of mutagenic activity have been found in both unconcentrated and concentrated samples in several advanced wastewater treatment plant effluents and intermediate points. The classes of compounds containing mutagens frequently detected were: weak acids, basic, ether insoluble, water soluble, aliphatic and aromatic neutrals, and many highly polar compounds. It appeared that some mutagenic activity increased during certain treatment steps. Wastewaters without industrial wastes and strictly of domestic origin did not have mutagenic activity in the tests.

Not all of the active compounds could be recovered from the wastewaters, even by use of three separate concentration procedures: polyurethane foam plugs, XAD resin, and solvent extraction. In relative terms, polyurethane foam plugs collected fewer toxicants. The presence of toxicants and/or masking agents rendered it difficult to uncover all the mutagenic activity of the wastewaters. It is not known what fraction of the total mutagenic activity of wastewaters has been uncovered in this investigation.

This study has provided a preliminary assessment regarding the mutagenic activity of renovated wastewater. Any consideration of the use of highly treated wastewaters for potable purposes must include a toxicological examination of such waters.

SECTION 3

RECOMMENDATIONS

The objective of the present investigation has been to assess, in a preliminary manner, the potential hazards linked to potable use of reclaimed wastewater. This investigation should be of value in identifying problem areas and in setting priorities for in-depth and more specialized toxicological and analytical studies. For example:

1. This study has shown that some in vitro bioassays are more suitable than others for assessment of mutagenic potential of wastewaters and renovated wastewaters. Some test organisms show greater susceptibility towards toxicants/masking agents, while others are not adequately sensitive to detect mutagens. An attempt should be made to validate assay systems for detection of mutagenic compounds in wastewaters, forming an emperical correlation between mutagenic activity and carcinogenic potential, and rank the assay systems based on their efficacy, sensitivity, and reliability for detection. Such a study should be performed utilizing wastewaters which have been spiked with known mutagens and carcinogens or their nonactive analogs.

2. The observed increase in mutagenic activity after liming and recarbonation, and certain other treatment steps, should be confirmed using other bioassay procedures. Efforts should also be made to uncover the mechanism responsible. If synthesis of mutagens takes place during treatment, identification of the precursors and definition of conditions that favor their synthesis should be of value in controlling their synthesis.

3. Efforts should be directed to devising new and more effective procedures for recovering mutagens from wastewater. A method that can be regarded as suitable for studies with wastewater should be able to recover a wide variety of mutagens from wastewater without recovering large quantities of toxicants. Perhaps the use of bioaccumulators (e.g. certain aquatic organisms) may offer this selectivity.

4. Studies should be undertaken to identify the chemicals responsible for the mutagenic activity and determine the concentration in the separated fractions.

5. This study concentrated on stable nonvolatile organics present in wastewater, and did not deal with information concerning the presence of low molecular weight volatile organics with mutagenic activity. Information on this class of compounds should be obtained.

SECTION 4

LITERATURE REVIEW

A literature review is presented here concerning the status of wastewater reuse in the United States and the potential health hazards associated with the presence of stable organics in renovated wastewater. A brief description of the most commonly used advanced wastewater renovation techniques is also given. In addition, a discussion is presented on the suitability of in vitro mutagenicity assays for studies with wastewater, and the ability of the bioassays to reveal quantitative information regarding human risk. It is felt that the information in these areas will help establish a basis for the study performed.

MUNICIPAL WASTEWATER REUSE IN THE United States

The total reuse volume in the United States at the present time is estimated to be approximately 248 billion gallons annually (Wesner and Hughes, 1979), and the interest in water reuse is continually increasing. Reuse of municipal wastewaters is being practiced at more than 536 locations in the United States (Wesner and Hughes, 1979). A majority of the reuse operations are located in the semi-arid southwestern United States.

The largest fraction of wastewater reuse is for irrigation (Table 1). The direct reuse of reclaimed wastewater for domestic use is very limited in the United States. The only current example of the direct reuse for nonpotable domestic purposes is in Grand Canyon Village, Arizona, where about 30,000 gallons per day of treated municipal wastewater are used for such purposes as toilet flushing and car washing.

TABLE 1. REUSE VOLUMES IN THE US AT THE PRESENT TIME
(Wesner and Hughes, 1979)

Type of Reuse	Volume in Billion Gallons
Agricultural Irrigation (landscape unspecified)	153.5
Industrial Process (cooling, boiler feed)	78.2
Groundwater Recharge	12.4
Other (recreational: fish and wildlife, etc.)	3.6
TOTAL	247.7

The alternatives to the direct reuse of renovated wastewater are: groundwater recharge either by surface spreading or by direct injection into an aquifer, or direct discharge of reclaimed wastewater for supply augmentation into a water supply system or storage facility. This is referred to as "intentional" indirect use. Water-short southern California is the area where "intentional" indirect use has had its most extensive application (Wassermann and Radimsky, 1979). The water supply authorities in the City of Denver are foreseeing the need to renovate wastewater for potable purposes in the future (Rothberg et al., 1979).

The unintentional reuse of wastewater also occurs widely. Rivers and lakes which serve as sources of potable water for public supply often serve as a medium for the disposal of treated and untreated wastewater. A limited amount of quantitative information regarding the extent of pollution of drinking water supplies by municipal wastewater has recently become available. For 1254 utilities using surface waters, Swayne et al. (1979) derived the ratio of municipal wastewater flow to streams or rivers. The results ranged from drinking water supplies with no identified discharges to many supplies where wastewater constitutes a major portion. During average flow conditions, up to 16% municipal wastewater was noted in the drinking water supplies of some utilities. Thus, through the water supplies, a portion of the public consumes some form of renovated wastewater every day.

ADVANCED WASTEWATER RENOVATION

Advanced waste treatment (AWT) refers to the removal of constituents beyond secondary waste treatment and involves removal of such items as phosphate, nitrogen, toxic metals, and organic material. It has recently been demonstrated that a large number of organic compounds are refractory to AWT, at least when they are present in trace amounts. Chappell et al. (1979) have analyzed both volatile and nonvolatile organics from wastewaters. The volatile organics were easy to identify by the purge and trap procedure, followed by analysis by gas chromatography. The nonvolatile organics, which have been reported to be 90% of the organics present (Aldridge et al., 1976), were difficult to trap because of the presence of both polar and nonpolar compounds. Also, analytical methods are not available to identify all nonvolatile compounds in a mixture. The effect of trace compounds in wastewater on human health will be discussed later.

Currently available AWT techniques fall into three categories: (a) biological, (b) physical-chemical, and (c) land applications. Some plants use a combination of these techniques.

Biological Methods

Biological methods used for the removal of carbon are similar to those which are used to achieve secondary treatment, for example, ponds, activated sludge, and trickling filters. However, in order to achieve high effluent quality, longer detention periods or improved liquid-solid separation techniques such as larger clarifiers or filtration units must be employed. The microbiological removal of nitrogenous compounds (ammonia, nitrites, organic nitrogen, and nitrate) involve nitrification followed by denitrification.

These processes can be accomplished in a mixed suspended culture or in a fixed medium, for example, biological growth supported on activated carbon (Jeris et al., 1971). Separate denitrification requires an organic carbon source, such as methanol, in order to carry out the reduction.

Biological processes have also been used for the removal of phosphorus from wastewater. This is based on the use of activated sludge microorganisms to take up phosphorus from inflowing wastewater, followed by subjecting the sludge to anaerobiosis to induce phosphorus release into a substream for chemical precipitation.

Physical-Chemical Methods

Physical-chemical methods are widely used in AWT for the removal of organics, nitrogen, and phosphorus. Passing the clarified water through columns filled with granular carbon is effective in removing a wide range of organic compounds and is an essential step in the production of potable water effluents. The physical-chemical techniques for removal of nitrogen include breakpoint chlorination, ion exchange, and ammonia stripping. Effluents containing less than 2.5 mg/l of total nitrogen have been produced by these techniques (EPA, 1975). Phosphorus is easily removed by precipitation with lime, ferric salts, alum, or alum salts. The precipitation can be carried out during primary sedimentation, in the secondary system, or as a separate final stage.

Several methods are available for the removal of dissolved salts from wastewaters. These include ion exchange, electrodialysis, distillation, and reverse osmosis with specially prepared membrane materials. The application of these methods, however, is currently limited largely to the industrial separation processes.

Disinfection is applied to some wastewater treatment plant effluents in the terminal stages to eliminate the microbial contaminants that have survived the various treatment processes. Chlorine is currently the most commonly used disinfectant. However, with recent findings that chlorination of water can result in the formation of new chlorine-containing compounds (Dowty et al., 1975; Glaze and Henderson, 1975), the use of alternate disinfectants is being considered.

Land Application Methods

Land treatment can be designed and operated as an AWT system. Municipal wastewaters, usually pretreated to some extent, are applied to land mostly by irrigation, overland flow, or infiltration-percolation (EPA, 1975). The treatment relies on microbial and chemical decomposition, adsorption on soil particles, plant uptake, volatilization, and leaching, and may require sizable land areas.

POTENTIAL HEALTH HAZARDS ASSOCIATED WITH THE PRESENCE OF ORGANICS IN RENOVATED WASTEWATERS

In the toxicological literature, few reports are available concerning

the long-term animal bioassays of renovated wastewaters and wastewater concentrates. In a preliminary investigation, Ottoboni and Greenberg (1970) found a significant reduction in the size of adrenal glands in female rats given filtered municipal wastewater ad libitum for 18 weeks. Some of the treated rats also developed tumors by six months of age. Spontaneous tumors are rare in this strain of rats at less than eight months of age. Trace organics present in the wastewater were implicated in these effects. At total organic carbon (TOC) levels up to about one thousand times the expected human exposure, recycled water from Blue Plains, Washington, DC, pilot AWT Plant produced only marginal toxicological and pathological effects in mice in a five-month feeding study (Gruener, 1978). The tests performed included: body weight gain, food intake, mutagenicity, mortality, blood physiology and biochemistry, liver and nervous system functions, and examination of tissues for pathological effects.

Health effect data, with respect to trace organics in reusable wastewater effluents, has been obtained recently with the help of rapid in vitro tests. Histidine requiring mutants of Salmonella typhimurium (Ames et al., 1975) have frequently been used in detecting the presence of mutagenic chemicals in recycled wastewater. Positive mutagenic response in these tester strains by various advanced wastewater treatment effluents and their concentrated organic fraction has been reported in numerous studies, among them: Saxena and Schwartz, 1979; Saxena et al., 1979; Rappaport et al., 1979; Pahren and Melton, 1979). Fractionation of positive organic concentrates (according to their solubility under acid, base, and neutral conditions) has shown the activity to be primarily in the basic and neutral fraction (Saxena et al., 1979; Rappaport et al., 1979). Gruener and Lockwood (1979) utilized V79 hamster cells (forward mutation) for the detection of mutagenic activity, and found a 20-fold increase in the mutagenicity rate over control with the organics recovered from the effluent of a pilot AWT plant. The organic mixture also induced a strong toxic response in human lung fibroblasts (WI 38) as revealed by steady loss of protein from cells. Marked inhibition of protein synthesis in cultured mouse fibroblasts (L-cells, Clone 929) was induced by concentrated samples of synthetic hospital wastewater after renovation by ultrafiltration, reverse osmosis, and ozonation (Cody et al., 1979). Deleterious effects of various chemical fractions of treated wastewater effluent separated by high pressure liquid chromatography were examined by Solomons (1979) by measuring the effect on intracellular adenine nucleotide pool in human neutrophils, monocytes, and platelets. The author reported a drastic reduction in cellular adenine nucleotide pool in the presence of several concentrated wastewater fractions. In the above-mentioned in vitro studies, the presence of toxicants, mutagens, and possibly carcinogens in reclaimed wastewater is indicated.

UTILITY OF IN VITRO SCREENING METHODS FOR ASSESSMENT OF MUTAGENIC POTENTIAL OF RENOVATED WASTEWATERS

In general, the approaches available for assessment of adverse human health effects linked to the consumption of renovated wastewater are: (a) epidemiological studies; (b) mammalian bioassay systems; and (c) in vitro bioassay systems. A full investigation of the potential hazards from the consumption of renovated wastewater cannot be performed within the time-frame

permitted and with the limited resources available by reliance on human epidemiology or animal cancer tests alone. These methods, in addition to being prohibitively expensive, will simply fail to keep up with the enormous volume of testing that is required. These difficulties have led to the utilization of *in vitro* bioassays or a battery of assays in the toxicological evaluation of renovated wastewaters either as a substitute for the conventional long-term assays or for establishing priorities for in-depth and more specialized toxicological testing.

At the present time, there are recognized uncertainties regarding the efficacy of various *in vitro* assay methods for detecting mutagenic and carcinogenic substances in complex mixtures such as wastewater/renovated water. Some possible ambiguities are: (a) if a mixture is active in a particular bioassay, what is the probability that it contains a mutagen; or (b) if a mixture is nonmutagenic, what is the assurance that it does not contain masked mutagens? On the other hand, the efficacy, sensitivity, and reliability of the *in vitro* systems for detecting mutagens in pure form has been fairly well defined. The results show that many of the available assay methods are able to detect a wide variety of mutagens. For example, the Ames' *Salmonella* assay has been reported to detect most carcinogens as mutagens except for those which operate through mechanisms other than genotoxicity, (e.g., asbestos, hormones, diethylstilbestrol). Fortunately, these carcinogens constitute only a small number of the total. Measures such as improvement of tester strains, more effective metabolic activation systems, modification of the assay conditions, and the use of a battery of short-term tests are helping further to shorten this list.

Another problem linked to utilization of *in vitro* assay systems in the evaluation of wastewaters/renovated wastewater concerns interpretation of the results in terms of human hazards. Some progress in the direction of quantitative evaluation of human risk from *in vitro* test results has been made, and the results obtained thus far look promising. The most commonly applied and most widely accepted assay, the Ames' *Salmonella* assay, has received the most attention in this respect. McCann and Ames (1977) have calculated carcinogenic potency as TD50 (the daily dose which will give half of the experimental animals cancer in a lifetime feeding experiment), for a large number of carcinogens, from the published animal cancer data. The dose has been found to vary by more than a million-fold. A similar range has been found in mutagenic potency in the *Salmonella* test (Table 2). This similarity has important implications in terms of at least rough quantitative correlation between mutagenicity in the bacterial test and carcinogenicity in animals.

TABLE 2. THE RANGE OF MUTAGENIC POTENCY IN THE *SALMONELLA*/MICROSOME TEST

	Revertants/nmole*	Ratio
1,2-Epoxybutane	0.006	1
Benzyl chloride	0.02	3
Methyl methanesulfonate	0.63	105
2-Naphthylamine	8.5	1,400
2-Acetylaminofluorene	108	18,000
Aflatoxin B_1	7,057	1,200,000
Furylfuramide (AF-2)	20,800	3,500,000

*McCann and Ames, 1977

A quantitative relationship between carcinogenic and mutagenic potencies has been noted for a number of polynuclear aromatic hydrocarbons (PAH) (Teranishi et al., 1975). In this study, the mutagenic potential of PAH in the Ames' assay was correlated with the carcinogenic activity expressed as Iball Index, which is based on epithelial tumor incidence in mice (Iball, 1939). The data revealed a clear quantitative correlation between carcinogenicity and mutagenicity with the frameshift mutant TA1538 but not with TA1537. Work performed by Meselson and Russell (1977) suggests a good correlation between the two potencies across a broad range of chemicals. For a variety of compounds which were both carcinogenic in rodent assays and mutagenic in the Ames' assay, these authors found that carcinogenic potency was approximately equal to mutagenic potency (Figure 1) with the exception of some nitrosoamines for which the carcinogenic potency was considerably greater than the mutagenic potency. The carcinogenic potency in this study was defined as the daily animal dose which gave 50% cumulative single-risk incidence of induced cancer after two years of exposure, whereas mutagenic potency was defined as the reciprocal of the number of micrograms of compound giving 100 revertant colonies in the Salmonella test system.

Recent studies have also indicated that considerable refinement of in vitro bioassay techniques may be necessary, before they can be used to predict human health hazards, either qualitatively or quantitatively. In a comprehensive study by LaVoie and co-workers (1979), it was shown that mutagenic potency of structurally-related PAH in the Ames' Salmonella assay did not accurately reflect either tumor initiating activity or complete carcinogenicity. Similarly, Roszinsky-Kocher and co-workers (1979) demonstrated that the induction of sister chromatid exchanges in Chinese hamster bone marrow cells by selected PAH was poorly correlated with their carcinogenic activity in animals. As an illustration of the misleading results which can be obtained with bacterial mutagenicity assays, Andrews and co-workers (1978) examined the mutagenic activity in petroleum and coal tar products. These substances have a high content of benzo[g,h,i]perylene (a noncarcinogen) relative to benzo[a]pyrene (a potent carcinogen) content. However, since both compounds can show nearly equal mutagenic activity in Salmonella, an incorrect assumption concerning carcinogenic potential of the mixture could result.

From the information presented in this section, it is clear that although there are recognized difficulties at this time in interpreting in vitro mutagenicity/carcinogenicity data in terms of carcinogenic potential to humans, the efforts made thus far have yielded promising results, and that with continued effort, such an interpretation should be possible. Moreover, with carcinogenic chemicals, there is a general agreement that exposure to any levels should be avoided. Thus, even qualitative information, or data which provides only a rough idea of mutagenic potency, should be of value. This fact, combined with the fact that in vitro bioassays currently available are able to detect a large number of mutagens/carcinogens in the pure form and are rapid and inexpensive, renders these assays extremely valuable in the toxicological evaluation of wastewaters/renovated wastewaters. Toxicological literature reveals several examples where short-term tests have successfully predicted carcinogenicity of a number of important environmental chemicals with widespread human exposure. Examples include the flame retardant, tris,

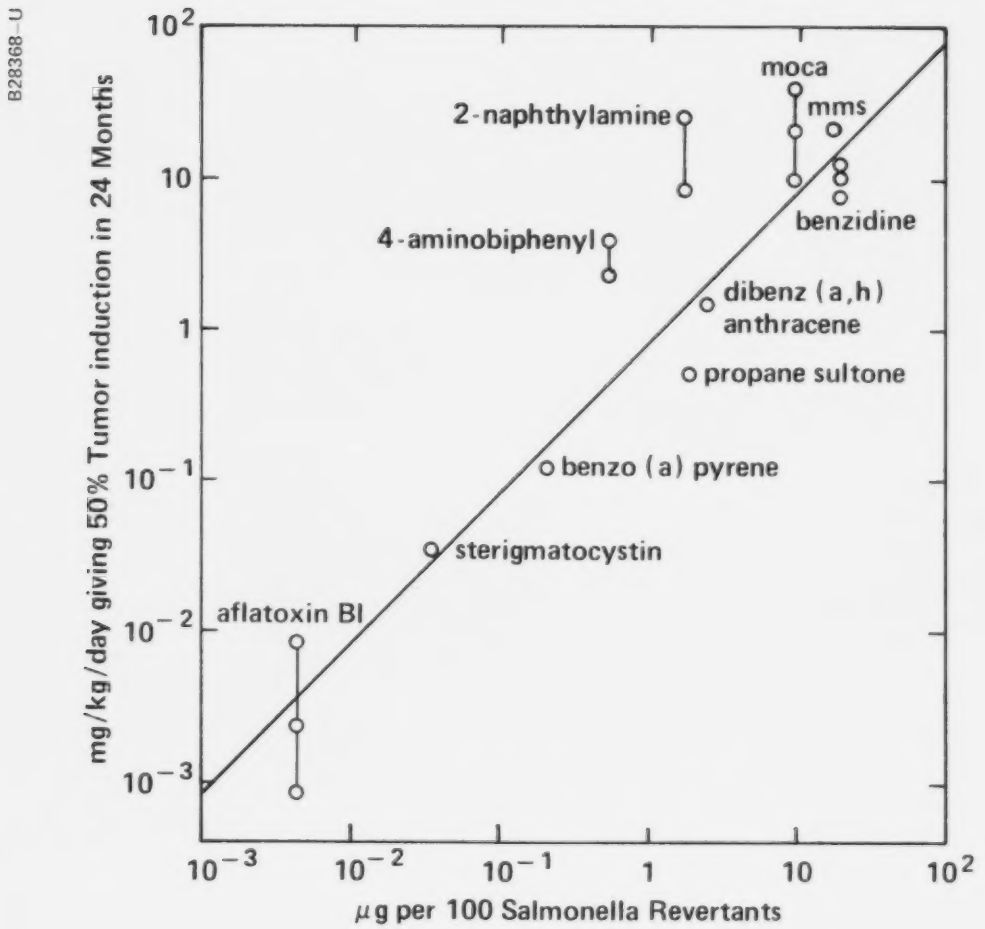

Figure 1. Relation between mutagenic and carcinogenic potency of ten compounds (Meselson and Russell, 1977)

the major industrial chemical 1,2-dichloroethane, and a series of hair dye chemicals.

SECTION 5

EXPERIMENTAL PROCEDURE

DESCRIPTION OF THE AWT FACILITIES

Plants representing a wide variety of treatment methods were selected so that the data on carcinogenicity/mutagenicity of the effluents could be related to treatment technology. It should be emphasized that none of these plants was designed to produce water for direct potable reuse and such use is not intended. However, since many of the same unit operations used in AWT processes would also be used in a process designed to produce potable water, it would be of interest to find out if mutagenic activity remains after advanced wastewater treatment. The salient features of the AWT plants/pilot plants selected are given below.

Piscataway (Maryland) Model AWT Plant (High Lime Process)

This is a five million gallon per day physical-chemical treatment facility. The treatment consists of lime additions, recarbonation (liquid carbon dioxide), dual media filtration, breakpoint chlorination, activated carbon filtration, and chlorination for disinfection. A flow diagram of the treatment steps used at this plant is shown in Figure 2. The flow received at this plant represents some industrial wastewater.

City of Niagara Falls, NY, Municipal Wastewater Treatment Plant

This is a physical-chemical treatment facility and is designed to handle 48 million gallons per day of combined industrial and municipal wastewater. Approximately 60%-70% of the wastewater flow is industrial. The treatment steps at this facility include removal of suspended solids and oxygen demand by the application of chemicals (lime and polymer) and by the physical processes of flocculation and sedimentation, then pH adjustment, activated carbon adsorption, and chlorination (Figure 3). A detailed description of the Niagara Falls AWT Facility is available in the paper by Sirianni and Marini (1974).

Bay Park Wastewater Renovation Plant East Rockaway, Long Island, NY

The secondary treatment system at this facility employed a two stage growth system with combined carbon oxidation-nitrification, intermediate clarification, denitrification, post aeration-stabilization and final clarification, and was based on the work of Jeris and co-workers (Jeris et al., 1971; Jeris and Owens, 1975). Methanol was used in the denitrification reactor as a source of supplemental carbon for the denitrification process. Following treatment with alum and polymer for secondary phosphorus removal,

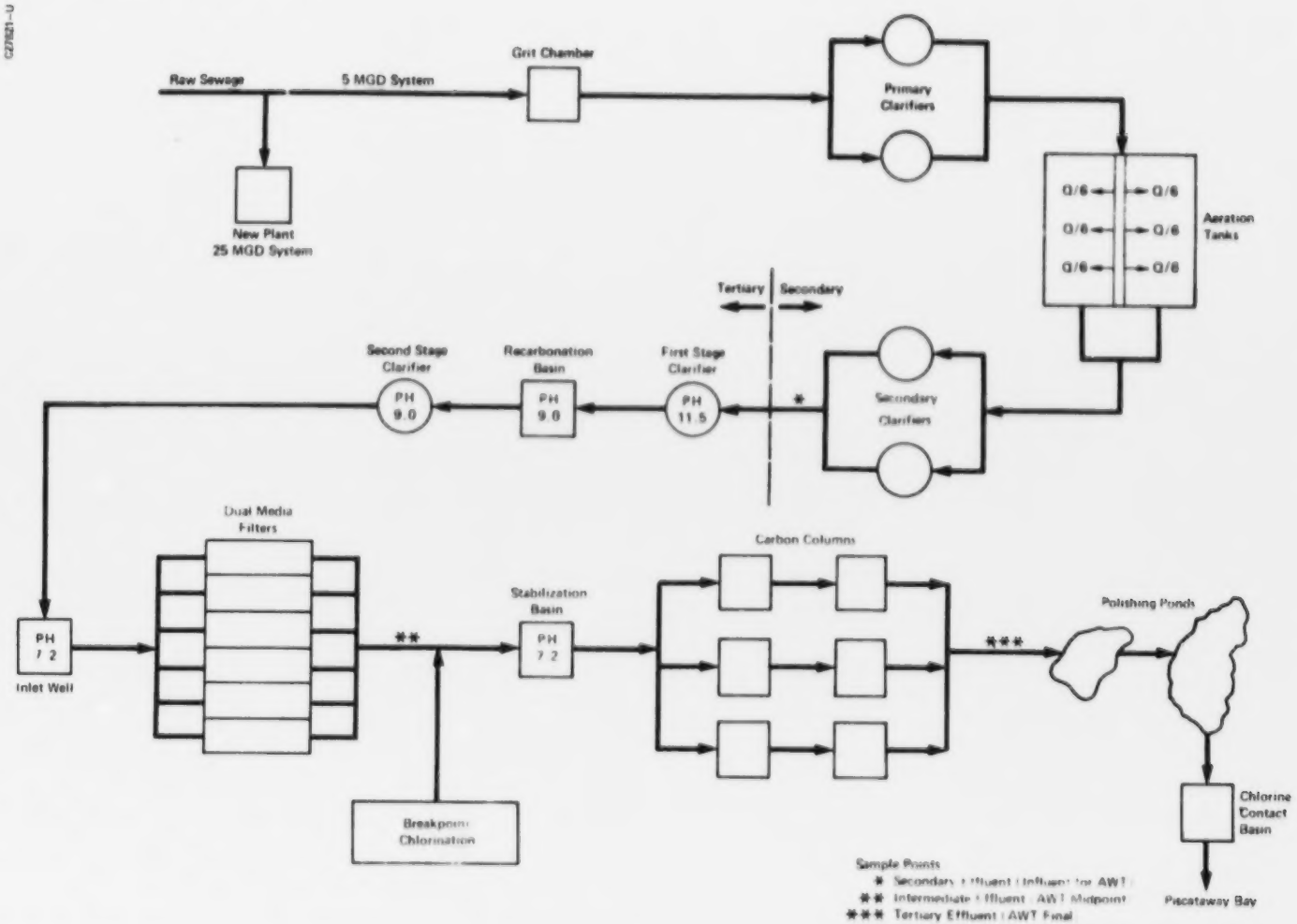

Figure 2. Advanced wastewater treatment at Piscataway, MD

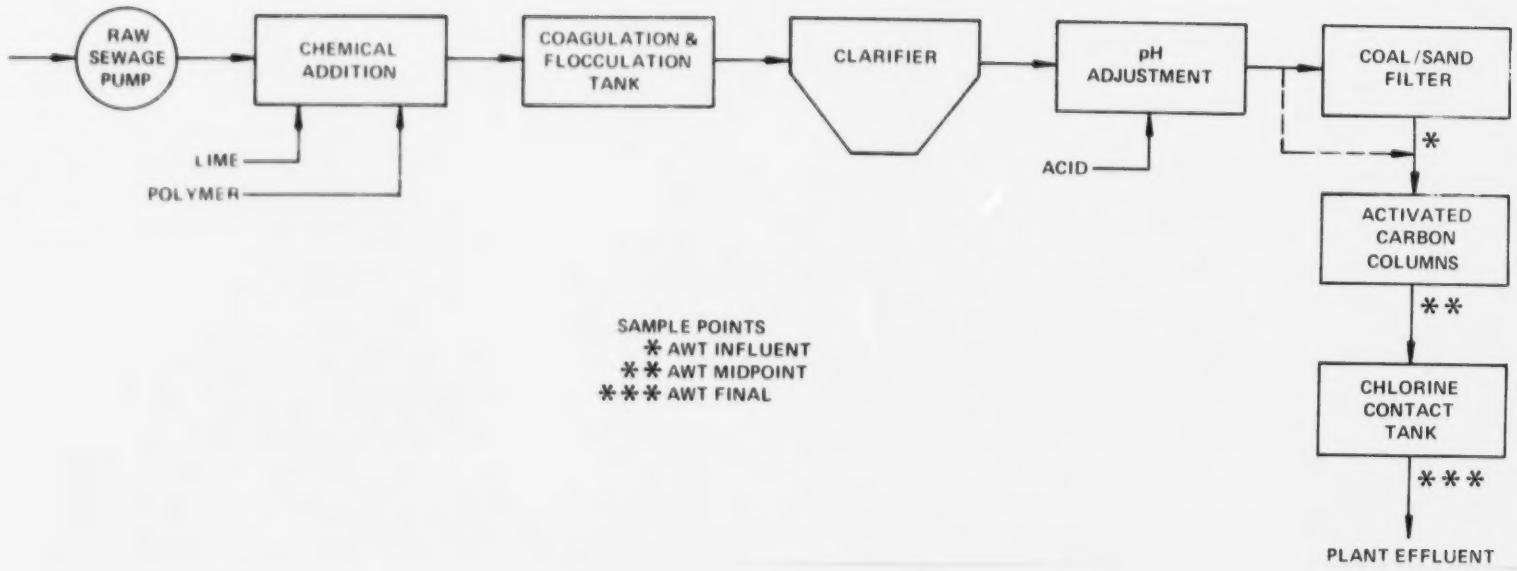

Figure 3. City of Niagara Falls Municipal Wastewater Treatment Plant

the effluent was passed through two gravity-type activated filters and then chlorinated.

The AWT effluent is used for groundwater recharge at 0.5 million gallons per day (Oliva et al., 1979). A schematic diagram of the water reclamation recharge facility is shown in Figure 4. The wastewater received by this facility is largely domestic. Changes were made at the BPWRP after our samples were taken. Therefore, results reported here may not be representative of present quality at the plant.

Lake George Village Sewage Treatment Plant

At this facility, the effluent from the conventional portion of the treatment plant, which consists of trickling filters followed by secondary settling, is applied to sand infiltration beds. The final effluents reach the groundwater. The treatment satisfactorily removes all the phosphorus and provides nitrified effluent that is believed to be safe for drinking (Aulenback et al., 1975). Although the Lake George Village Sewage Treatment plant (LGSTP) does not receive any industrial effluents and the presence of mutagens or carcinogens in domestic wastewaters would be unlikely, a rationale for its inclusion was to examine the possibility of microbial synthesis of such compounds during land filtration of wastewaters. Synthesis of strong mutagens by microbial action has been noted in other environments (Alexander, 1974).

For some studies, the rapid sand filtration process was simulated in the laboratory. The columns used were plexiglass cylinders, six inches in diameter, and approximately 14 feet in length. They were filled with between 9 and 12 feet of sand taken from the dosing bed at the LGSTP. Taps were placed in the columns at two-foot intervals and one at the bottom. The two columns used in this study represent the sand collected from the two different beds. Biological growth on the columns was reinstituted prior to their use by passing treated (LGVSTP) effluent through the columns for a period of two weeks.

SAMPLES AND SAMPLING PROCEDURE

Samples representing various stages of treatment were chosen. From the Piscataway and Niagara Falls plants, three samples were collected: the influent to the AWT process, intermediate stage effluent, and final effluent. In the case of Bay Park and Lake George plants, only influent and effluent samples were collected. Sampling points are shown in the flow diagram for each of the treatment facilities.

Assay of mutagenic/carcinogenic activity was performed both on unconcentrated waters and on the organic concentrates prepared from these waters. Procedural details pertaining to the sample collection and preparation of organic concentrates follow.

Unconcentrated Wastewaters

Twenty-four hour composite samples were collected in order to obtain a

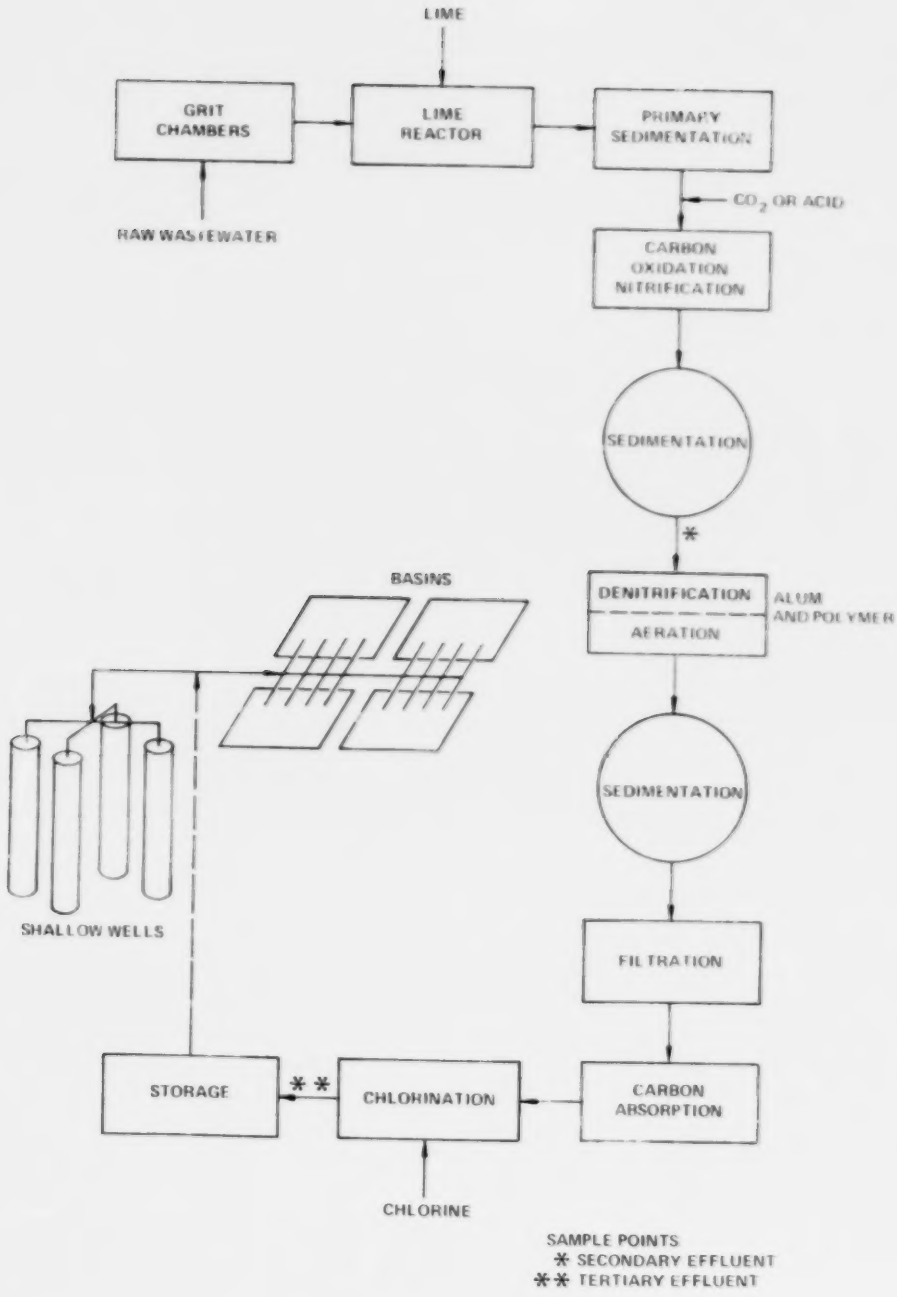

Figure 4. Schematic diagram of the Bay Park Wastewater Renovation Plant, East Rockaway, Long Island

sample representative of the full spectrum of waste entering the treatment plant on a given day. It was recognized that compositing might dilute the mutagenic substances present in one or several fractions of the sample. A similar dilution and mixing is, however, expected in a water reuse situation, and, therefore, a composite sample appeared to represent a more realistic situation. ISCO automatic composite samplers were used with a sample collection rate of approximately 250 ml every 30 minutes. The samples were kept in wet ice during the compositing period and transportation to prevent sample deterioration. In view of the generally recognized quality fluctuations of the influent wastewaters, two samples which were collected several weeks apart were tested. Samples were sterilized by serial filtration with 1200 nm 450 nm, and finally 200 nm membrane filter (Millipore Corporation).

An exception to the above procedure was employed for the collection of final effluent samples from the rapid sand filtration system at LGVSTP. These samples were collected from 25-foot and 60-foot depths of the sand bed with the help of porous cup lysimeters maintained under negative pressure. The samples were withdrawn from the lysimeters by application of positive pressure with a pump. The sample volume that could be collected from the lysimeters was small and ranged from 300 ml to 500 ml. This, and the fact that 5 to 10 days were required for the lysimeters to fill, rendered it necessary to pool the samples for a particular analysis.

RECOVERY OF ORGANICS FROM WASTEWATERS

A number of methods are available for removal of organic compounds from water, for example: adsorption on activated carbon (Braus et al., 1951); low temperature distillation, freeze drying (Pitt et al., 1975); (Katz et al., 1972); porous polyurethane foam columns (Gesser et al., 1971; Uthe et al., 1972; Basu and Saxena, 1978) organic resin adsorption on Amberlite XAD resin (Rohm and Haas Co.,) (Burnhan et al., 1972); and batch and continuous solvent extraction (Schaefer et al., 1968; Ahnoff and Josefsson, 1974). There are limitations with many of these concentration methods. Some are unattractive because of their inability to handle large sample volumes, while others require complex apparatus which cannot be easily used in the field. Also, no single concentration method could be used to recover the wide variety of organic contaminants present in renovated wastewater.

In view of these limitations, three concentration methods were chosen for use in this study based on their individual properties. The methods selected were: sorption on polyurethane foam plugs, sorption on XAD resin, and liquid-liquid extraction. Porous polyurethane foam is extremely efficient in collecting a number of environmentally significant classes of compounds such as polychlorinated biphenyls, chlorinated insecticides (Musty and Nickles, 1974), phthlate esters (Gough and Gesser, 1975), and PAH (Saxena et al., 1977). XAD resins have shown moderate efficiency for a broad range of nonionic organic compounds (Burnham, et al., 1972). Both XAD and polyurethane foam plugs allow high flow rates and can be used to recover organics from large volumes of water. Liquid-liquid extraction using 15% methylene chloride in hexane has been successfully used to extract many pesticides and other organic contaminants.

The concentration of raw organics from wastewaters by XAD and polyurethane foam was performed at the AWT site using a portable apparatus which allowed for the maintenance of optimum conditions for recovery of organic compounds. After sample collection, the sorbent material was brought to the laboratory for further processing. This helped overcome the difficulties associated with shipping large volumes of water for concentration, and possible deterioration of samples during transportation and storage. For liquid-liquid extraction, composite samples were brought to the laboratory. The details of each concentration method are given below.

Recovery of Organics by Sorption on Polyurethane Foam Plugs

The procedure for recovery of organics by sorption on polyurethane foam plugs involved heating the wastewater to 62°C and passing it through the foam plugs at a flow rate of 250 ± 10 ml/min (Saxena et al., 1977). Four foam plugs were used for recovering organics from 10ℓ of secondary effluent, and 20ℓ of midpoint and final effluent. The wastewaters were filtered through glass wool prior to passing through foam plugs to prevent clogging. Organics from a total of 60ℓ of secondary and 120ℓ of midpoint and final effluents were collected. It was assumed that the levels of active compounds in secondary effluent would be relatively high; therefore, concentration of 60ℓ would be adequate.

A modification of the previously developed field sampling apparatus (Saxena et al., 1977) was used for concentrating organics from wastewaters. These modifications included: the incorporation of a peristaltic pump (Cole-Parmer Instruments Inc.) for more reliable and constant flow rate; the inclusion of a water meter (Kent Meter Sales, Inc.) for measurement of sampling volume; and the assembly of the sampling apparatus into a suitcase to facilitate its transportation and field set up.

The sorbed compounds were eluted from the foam plugs by extraction with 45 ml acetone and 125 ml cyclohexane. The extract was shaken, and on separation of phases, the bottom acetone-water phase was removed and discarded. The cyclohexane phase was dried with anhydrous sodium sulfate and evaporated to dryness using a rotary evaporator at a temperature of 40°C. The residue was reconstitued with dimethylsulfoxide for use in the bioassay systems.

Liquid-Liquid Extraction Procedure

For the recovery of organics by liquid-liquid extraction, 24 hour composite samples were brought to the laboratory. The samples were preserved in transit by the addition of 1 ml methylene chloride/ℓ of wastewater. Organics were recovered from the wastewater by extraction for 24 hours with 15% methylene chloride in hexane (75 ml solvent/ℓ wastewater). The organic phase was separated, dried by passing through a column of anhydrous sodium sulfate, evaporated to dryness, and reconstituted in dimethlsulfoxide as described earlier.

Recovery of Organics by Sorption on XAD Resin

XAD resins have been used extensively for the collection of trace

organics from surface waters Junk et al., (1976) and drinking waters (VanRossum and Webb, 1978). The conditions for collection of organic material from these waters have been defined, little work has been reported, however, regarding collection of organics from wastewater. Municipal wastewater contains higher levels of dissolved organics and particulate matter than either surface water or drinking water, and, therefore, it was considered essential to determine the parameters necessary for the efficient collection of organics from this source. The objective of this study was not to perform an in-depth investigation of the sorption characteristics of XAD but merely to define sampling parameters for collection of organics from wastewater. Thus, the study was performed in a preliminary manner, and the time devoted was kept short.

The most widely used resin XAD-4 (Rohn and Haas Co.) was used in these studies. Prior to use, the resin was cleaned by successive Soxhlet extraction for 22 hours with methanol, acetonitrile, and diethyl ether. The purified XAD resin was added to a column (1 X 30 cm) as a methanol slurry until a resin bed of approximately 10 cm high was obtained. Methanol was drained and the column was washed with approximately 600 ml of distilled water. After passing wastewater through the column, the retained organics were eluted with 50 ml methanol. The methanol was evaporated, and the organic residue was quantitated gravimetrically.

Methanol was chosen as a solvent for elution, because of its superior performance over acetone, acetonitrile, and diethyl ether. To determine the efficiency of these solvents for eluting organics sorbed on the resin, equal aliquots of the resin previously exposed to wastewater were eluted with different solvents in Soxhlet apparatus. The organics recovered with each solvent were quantitated. The elution efficiency of methanol, acetone, acetonitrile, and diethyl ether is shown in Table 3.

TABLE 3. COMPARISON OF ELUTION SOLVENTS FOR USE WITH XAD RESINS

Solvents	mg of Organic Matter Extracted/20 g of Resin
Methanol	42.4
Acetone	36.8
Acetonitrile	33.8
Diethyl Ether	27.4

Further studies were carried out to define the optimum flow rate, wastewater temperature, and the breakthrough volume for the recovery of organics from wastewater. Flow rates in the range tested (50 to 100 ml/min) showed no significant effect on column efficiency. Increase in wastewater temperature, on the other hand, resulted in an increase in the retention efficiency. At a temperature of 50°C the increase in column retention efficiency was approximately 20% over the efficiency obtained at 20°C. In

order to determine the breakthrough volume, increasing volumes of wastewater up to 9ℓ were passed through the XAD column at a temperature of 50°C. Volumes greater than 9ℓ could not be passed because of the clogging of the column. The column retention efficiency in these studies was quantitated based on the fluorescence of influent and effluent (excitation wavelength 275 nm peak emission wavelength 365 nm). The results showed that the efficiency of retention for organics steadily declined as the sample volume increased. The efficiency of retention with 2, 5, and 9ℓ of wastewater was, respectively, 21%, 14%, and 12%.

XAD-4 is a hydrophobic adsorbent and may not efficiently recover all the compounds. In an attempt to recover the classes of compounds which are inadequately recovered by XAD-4, the possibility of using a column of XAD-8 in series with XAD-4 was investigated. XAD-8, being an adsorbent of intermediate polarity or hydrophilicity, is expected to have greater affinity for polar compounds compared to XAD-4. The addition of a column of XAD-8 with XAD-4 enhanced the retention efficiency by approximately 28% over the retention efficiency of XAD-4 alone.

From the results reported above, the suitable conditions for collecting organics from wastewater were determined to be as follows: wastewater temperature 50°C, flow rate 100 ml/min, XAD-4 and XAD-8 columns (1 X 10 cm) connected in series and a sample volume of 5ℓ. The total organic carbon retention under these conditions ranged between 18% to 20% for the local secondary effluent with an initial TOC of 35 ppm. The sampling apparatus described earlier for the recovery of organics by polyurethane foam plugs, was used for maintaining the above-mentioned sampling conditions in the field with XAD columns. The compounds sorbed on the resin were recovered by Soxhlet extraction for 18 hours with methanol. The eluate was solvent exchanged with dimethylsulfoxide (DMSO) for bioassay.

EVALUATION OF SORBENT MATERIAL AND SOLVENTS FOR POSSIBLE CONTRIBUTION OF MUTAGENS/CARINOGENS

Prior to utilizing polyurethane foam or XAD resin for preconcentration of organics from renovated wastewater, it was important to discover if the sorbents themselves would leach out any toxic and/or mutagenic material. It was equally important to ascertain the presence of toxic and/or mutagenic impurities in the solvents used for liquid-liquid extraction of wastewaters and for elution of organics from solid sorbents that may get concentrated during solvent volume reduction.

For these studies, foam and XAD columns without exposure to wastewaters were eluted, the eluate evaporated to dryness, and the residue resuspended in DMSO. The residues remaining after evaporation of the test solvents were resuspended in DMSO in a similar manner and subjected to the bioassays with and without mammalian metabolic activation. The concentrations of the extract in the bioassays were kept several-fold higher than that which would normally be present. The study revealed no noticeable mutagenicity/carcinogenicity or toxicity resulting from foam, XAD extract, or solvent residues.

CHEMICAL SEPARATION OF ORGANIC MIXTURES INTO MAJOR CHEMICAL CLASSES

Renovated wastewater concentrates were chemically fractionated and each fraction was tested for mutagenic potential. The objective of this study was twofold (a) identify chemical class(es) in the crude organic mixture responsible for the mutagenic activity, and (b) uncover the activity which may have been masked because of the complexity of the concentrates. It was believed that chemical fractionation would result in enrichment of mutagenic substances in selected fractions separated from toxicants thereby facilitating their detection by *in vitro* bioassays.

The crude organic mixtures recovered from wastewater were separated into major classes of organic compounds according to their solubility under acid, base, and neutral conditions using a modified Shriner-Fusion separation technique (Breidenbach *et al.*, 1966). The mixture representing 12ℓ of wastewater was brought to dryness, resuspended in ethyl ether, and separated into seven chemical classes: ether insoluble, water soluble, basic, amphoteric, strong acid, weak acid, and neutral. Adsorption chromatography with silica gel column was used to subfractionate the neutral fraction into aliphatic, aromatic, and oxygenated fractions. The neutral fraction representing 12ℓ of wastewater was adsorbed onto approximately 1 g of silica gel and applied to the top of the column. The column was eluted successively with isooctane, benzene, and a chloroform and methanol mixture (1:1) to elute aliphatic, aromatic, and oxygenated compounds, respectively. Radiolabelled benzo(a)pyrene was used to test the performance of the column and of the elution procedure. The complete separation scheme is shown in Figure 5.

Separated fractions were stored under refrigeration until needed for the bioassays, the desired quantity was removed and reduced to dryness under nitrogen. The residue was redissolved in DMSO.

Little is known concerning the chemical composition of the separated classes of compounds. Some generalizations can be made regarding the type of compounds that may be expected to be present in these fractions, based on the studies performed on other complex environmental mixtures, for example air particulates and tobacco tar (Hoffman and Wynder, 1968, 1971). The strong acid fraction is expected to contain acidic compounds that form salts with weak bases, such as carboxylic acids and some fatty acids. The compounds in the weak acid fraction need a strong base to form a salt, such as phenolic compounds. The basic fraction which forms salts with strong acids, is expected to contain compounds such as amines. The neutral fraction consists of three subclasses: aliphatic, aromatic, and oxygenated. The aliphatic fraction, which elutes with isooctane from silica gel, contains saturated and unsaturated hydrocarbons. Although most known compounds in this class are biologically inactive, there are indications that some paraffins may be tumor-promoters (Horten *et al.*, 1957). The second subclass which elutes with benzene has been shown to contain members of the polynuclear family. Several of these compounds are known animal carcinogens (Santodonato *et al.*, 1979). The oxygenated fraction may contain epoxides, peroxides, aldehydes, ketones, and lactones. Some of the oxygenated compounds are also known to be carcinogenic.

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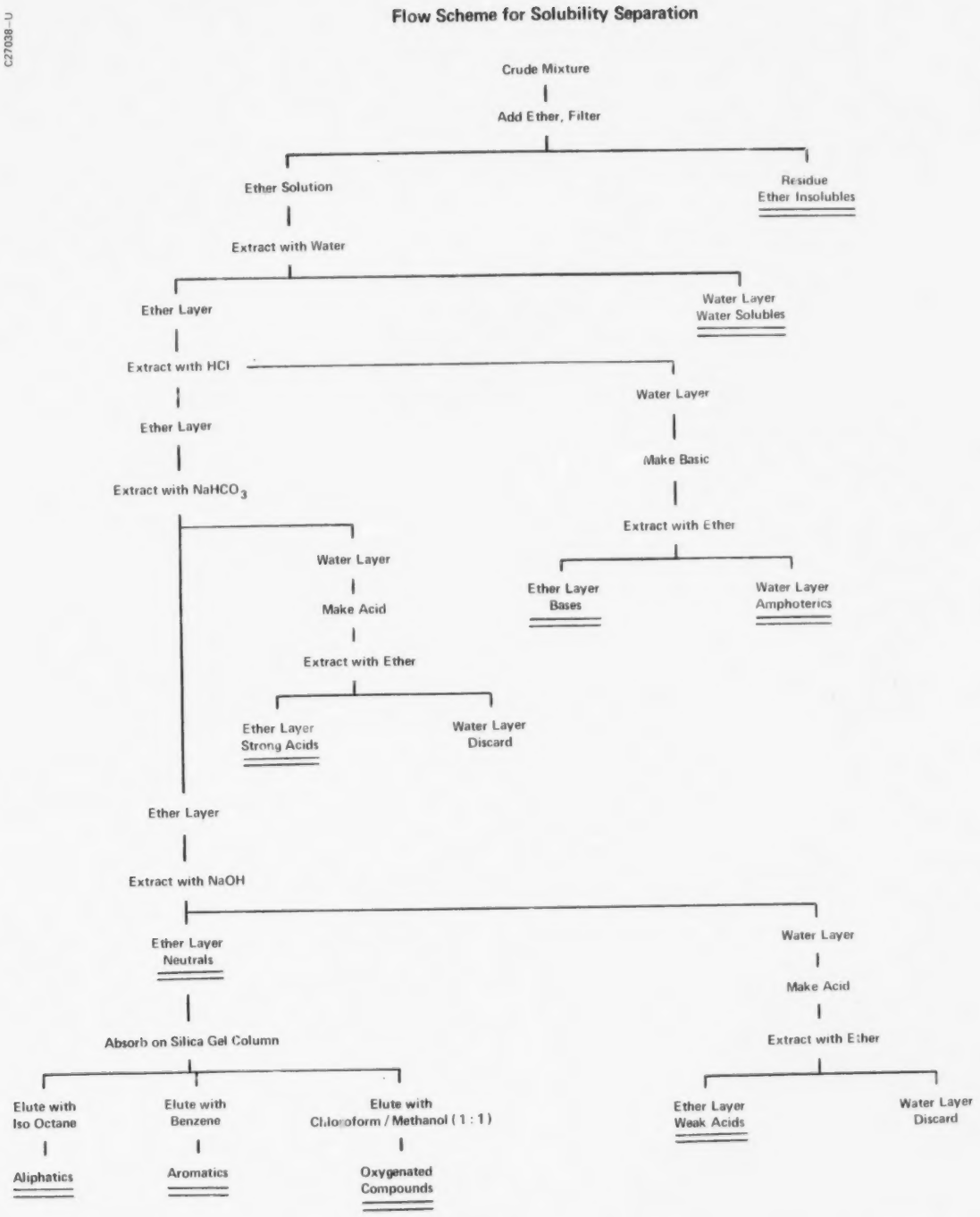


Figure 5. Flow scheme for solubility separation

TOTAL ORGANIC CARBON DETERMINATION

Unconcentrated wastewater, crude organic concentrates, and separated fractions were quantitated by determination of TOC. TOC content was also used as a guide to determine the concentrations to be used in the bioassay. TOC analysis on unconcentrated wastewater was performed on glass wool filtered wastewater using the direct injection mode of the Oceanography International Corporation's TOC analyzer. Nonvolatile TOC of the organic concentrates and separated fractions which were dissolved in organic solvent were determined using the ampoule module of the TOC analyzer (wet oxidation method). The test sample of organic concentrate was placed in a glass ampoule and the solvent vehicle evaporated by incubation at 60°C overnight. Any residual solvent vapor was removed by flushing the ampoule with nitrogen. The residue was reconstituted in distilled water. The rest of the analysis was completed in accordance with the standard oxidation method described for aqueous samples.

ASSAY OF MUTAGENIC/CARCINOGENIC ACTIVITY

Rapid in vitro bioassays were used in these studies. In vitro assays in general have the advantage of speed, ease of testing, low cost, and generation of fairly precise results. An assay system chosen for studying mutagenic potential of recycled wastewater should not only detect mutagens with a high degree of accuracy but should be able to do so in the presence of the materials generally present in wastewater. Wastewater, like any complex environmental sample, contains mutagenic and carcinogenic substances in a matrix of innocuous and toxic substances. These substances may inhibit, or modify, the response of the assay system. In the absence of any information regarding performance of various available assay methods with wastewaters, it was considered safer to use a battery of assay systems. Also, with the utilization of several test methods, the results obtained would be far more reliable. The three assay systems chosen for this study were: Ames' Salmonella assay, Saccharomyces mutagenesis assay, and cell transformation assay with BHK_{21} Cℓ 13 cells. The Ames' assay and BHK_{21} Cℓ 13 cell transformation assays were selected based on their high sensitivity and efficiency of detection for chemical mutagens and carcinogens in pure form. An assay involving diploid yeast (Saccharomyce cerevisial was included in the study, because this is the only practical and rapid assay for somatic recombination and gene conversion. These categories of genetic damage are important from the point of view of human health. Moreover, yeast offers an opportunity to study a number of genetic parameters such as forward and reverse mutagenesis, inter- and intra-allelic recombination, and chromosomal aberrations in the same organism. A flow diagram of the bioassay procedure is presented in Figure 6.

Ames' Salmonella Assay

This assay utilizes histidine requiring strains of S. typhimurium specially constructed by Ames and co-workers and has the potential to detect carcinogens as mutagens (Ames et al., 1975). This assay can detect point mutations of both frameshift and base-pair substitution mutations, is easy to perform, and has the capacity to approximate mammalian metabolic activation. In the present studies, Salmonella strains TA100 and TA1535 were used as

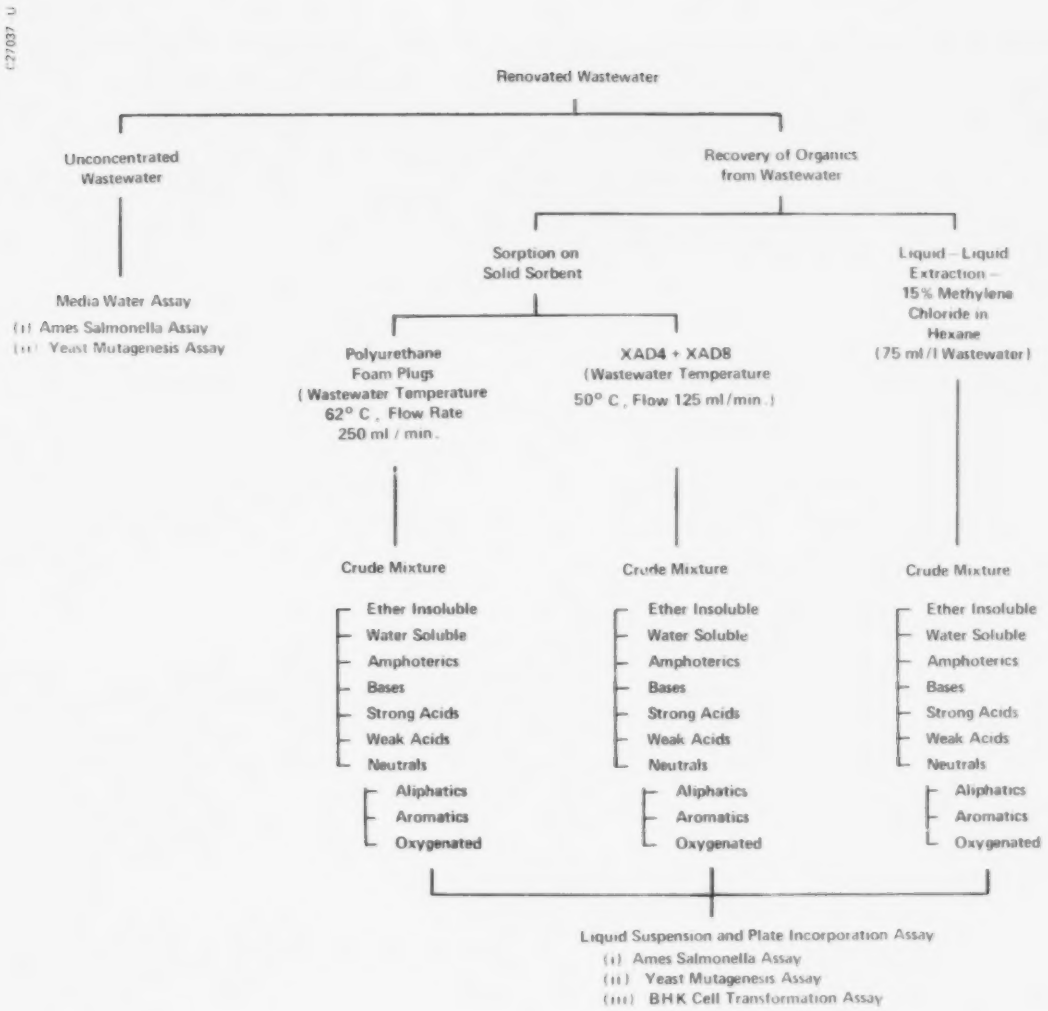


Figure 6. Overall protocol followed for mutagenicity/carcinogenicity testing of renovated wastewater

indicators of base pair substitution mutation, and strains TA98 and TA1538 as indicators of frameshift mutation. The assay was performed both in the presence and absence of mammalian metabolic activation system. Experimental details of the assay method are given below.

Preparation of Cell Suspension--

Suspensions of Salmonella tester strains for the assay were prepared using a modified procedure. Instead of growing fresh cultures every day for mutagenesis studies, a method was devised for storing cell suspensions without loss of viability or mutagen sensitivity for a period of up to two months. The procedure involved growing large batches of cells, harvesting the cells by centrifugation, and resuspending them in nutrient broth supplemented with 20% glycerol (a cryoprotective agent). The cell population was adjusted to 5 X 109 cells/ml using a previously established optical density at 660 nm versus cell population relationship. Small aliquots of the suspension were frozen at -65°C. Cell suspensions were thawed on the day of the experiment and used at 0.1 ml/agar-overlay in the mutagenesis assay. This modification provided consistently reproducible bacterial growth on petri dishes (background "lawn") and eliminated the problem of sparse "lawns" which arises with slow-growing strains of Salmonella. This led to significant improvement in the reproducibility of the Ames' assay and saved time.

Spontaneous Reversion Rates for the Tester Strains--

Spontaneous reversion rates for the tester strains are shown in Table 4. The data shown are derived from spontaneous reversion frequencies observed in duplicate from five sample runs. It should be noted that experimental results given later in the report were compared to their own individual control values and not the calculated mean given in the table.

Diagnostic Mutagens Used--

The performance of the mutagenesis assay was routinely checked using diagnostic mutagens as positive controls. Diagnostic mutagens not requiring metabolic activation included: nitrosoquanidine (2 µg/plate), bis(4chlorobutyl) ether (100 µg/plate), and methylmethanesulfonate (50 µg/plate) and those requiring metabolic activation: benzo(a)pyrene (5 µg/plate), 2-aminofluorene (10 µg/plate), and aflatoxin B_1 (1 µg/plate).

Mammalian Metabolic Activation System--

Metabolic activation of mutagens/carcinogens in the assay was achieved by incorporation of liver homogenate from rats induced with polychorinated biphenyl (PCB). The procedure, reported by Ames et al. (1975) for preparation of rat liver homogenate and liver homogenate-cofactor mix was used without any modification. Each batch of rat liver homogenate was checked for bacterial contamination and for activity with diagnostic promutagens. Studies were also conducted to determine if the chemical contaminants present in wastewaters/renovated wastewaters could serve as liver enzyme inducing

TABLE 4. SPONTANEOUS REVERSION FREQUENTY OF THE SALMONELLA TESTER STRAINS

Parameters	Spontaneous Reversion per 10^9 Bacteria/Strain									
	TA98		TA100		TA1535		TA1537		TA1538	
	-	+	-	+	-	+	-	+	-	+
Mean	27.38	38.6	117.8	178	49.8	50	14.4	31.2	14.5	28.4
Standard Deviation	±10.23	±6.21	±44.48	±34.30	±11.65	±13.74	±9.94	±17.85	±6.41	±5.68
Range	18-42	31-45	141-251	141-234	35-62	35-61	8-30	10-52	7-23	20-36
Number of Samples	6	6	5	5	5	5	5	5	6	5

agents. These studies were conducted using wastewater from the Bay Park and Piscataway AWT Plants. Rats were orally given test wastewaters ad libitum for five days, following which liver homogenates were prepared. The homogenates were tested for activity in the Ames' Salmonella assay with known promutagens, and the activity (reversion rate/mg homogenate protein) was compared with that obtained with Aroclor injected and corn oil injected control rats. The results showed no significant increase in activity in the rats given wastewater, over corn oil treated rats. This suggested that wastewaters did not contain inducing agents in sufficient quantities. Alternately, the time of exposure to wastewater may have been too short to see an effect. It is also possible that an increased activity may not have been observed due to the presence of inhibitors of activating enzymes in the wastewaters.

Assay of toxicity--

Toxicity assays with wastewater/renovated wastewater concentrates were carried out to determine the concentration range to use in the mutagenicity studies. In these studies, 200 cells were plated on nutrient agar in the presence of various amounts of test material. Following 48 hours of incubation, the number of colonies on the plates were counted, and results compared with untreated plates.

Variations of the standard mutagenicity assay used--

Two variations of the standard plate incorporation assay were used in assessing the mutagenic potential of wastewater. The first, designated as "media water assay," was used with unconcentrated wastewater samples. The second method, designated as "liquid suspension assay," was utilized with crude organic concentrates of wastewater and separated fractions. Experimental details of the assay methods are given below.

Media Water Assay--To facilitate detection of low concentrations of mutagens suspected in unconcentrated wastewater, the bioassays were performed by making filter-sterilized test waters into complete media (base agar layer). Different strengths of wastewater in the media were obtained by dilution of test wastewater with an appropriate volume of distilled water prior to preparation of the media. Tubes containing soft agar and histidine-biotin were supplemented with 0.1 ml of the cell suspension and 0.5 ml S9 mix (where added), were overlaid on the base agar layer. Thus, suspected mutagens would be present in the bottom agar, whereas the test organism and mammalian activation system would be in the agar overlay. Controls were overlaid on base agar layer made with distilled water.

The above modification of the assay permitted incorporation of up to 20 ml test wastewater/assay (70% v/v) and was particularly valuable in the situations where the concentration of suspected mutagen was so low that large volumes of sample must be incorporated in the assay. To increase reliability and facilitate comparison of the data, assays on all the samples collected

from one location were performed at the same time along with distilled water controls for spontaneous reversion rate and positive mutagenesis controls. In order to substantiate the expected low reversion rates with unconcentrated wastewaters, efforts were made to obtain dose response data. The volumes of wastewater tested were, 1, 10, and 20 ml/assay. While 10 and 20 ml wastewater/assay were evaluated using the media water assay, the plate incorporation assay was used in studies with 1 ml. The results are expressed as averages of triplicate determinations.

Using known mutagens, it was shown that the mutagens added to the bottom agar were detected with lower efficiency than those added to the top agar. For example, benzo(a)pyrene, an extremely hydrophobic molecule, was detected in the media water assay with 10 times less sensitivity compared to occasions when the mutagen was added to the top agar. Thus, the actual mutagenic response with the wastewaters should be higher than that reported here with the media water assay.

Liquid suspension assay--This assay involved incubaton of the test mixture and indicator organisms in liquid suspension prior to plating and was particularly valuable with complex uncharacterized mixtures where the possibility exists that some of the compounds present (or a metabolically active form) might be inactivated by agar. In addition, the assay possessed all the features of the plate incorporation assay and could easily detect every compound which the conventional plate incorporation assay was capable of detecting. DeSeores and Shelby (1979) have recommended this procedure in cases where results from the standard plate incorporation assay are inconclusive.

Preliminary studies showed that many of the crude organic concentrates and separated fractions which were negative in plate incorporation assays showed mutagenicity readily in liquid suspension assays. Therefore, the liquid suspension assay was routinely used in assessing the mutagenic potential of wastewater concentrates and separated fractions. The preincubation medium for the assay contained 1 ml minimal medium, 0.5 ml rat liver homogenate plus cofactors (S-9 Mix) where added (Ames et al., 1975), 0.1 ml appropriate tester strain (5×10^8 cells), 4 µg histidine and 6 µg biotin, and appropriate amounts of the test organic mixture. Identical control tubes were set up without the test organic mixture. Following a 60 minute incubation at 37°C, 3 ml soft agar, containing 24 µg histidine and 36 µg biotin/ml, was added and the contents were overlaid on minimal glucose agar. Mutagenicity was shown by an increase in the number of revertants over spontaneous (control). The assays were carried out in duplicate.

Assay of Histidine in Wastewaters--

The amino acid histidine which interferes with Ames' assay by artifactually increasing the number of revertants, is routinely found in domestic wastewater (Faust and Hunter 1971). Studies with minimal glucose agar supplemented with increasing concentrations of histidine revealed that the number of revertant colonies was directly proportional to the histidine concentration. Thus it was important to determine if histidine was present in the wastewater samples.

Wastewaters were assayed for the presence of histidine using an enzymatic assay which involved conversion of histidine to urocanic acid by L-histidine ammonia lyase (E.C.4.3.1.3). The reaction was followed by increase in absorbance at 277 nm (Hassall, 1971). No histidine could be detected in any of the wastewaters studied. The detection limit of the method was 1 µg/ml.

Derivation of Results--

In studies with unconcentrated wastewaters, any reproducible increase in the number of revertants in the presence of test wastewaters over spontaneous was regarded as a mutagenic response. On the other hand, in plate-incorporation studies with wastewater concentrates and separated fractions, a ratio of colonies (treated/spontaneous) of 1.5 to 2.0 was regarded to be a weak mutagenic response, and a ratio of 2.0 and higher as a mutagenic response. These criteria used for interpreting the results are different from those conventionally used with pure compounds where a minimum of doubling in the number of revertants is regarded as positive. The criteria generally agreed upon with pure compounds could not be adhered to in the studies with unconcentrated wastewater because of the low expected mutagen concentrations or in studies with crude concentrates and separated fractions because of the interference caused by toxicants present in the test materials. A detailed background to the problem of mutagenicity assay of wastewaters is presented in the Result and Discussion Section.

Saccharomyces Cerevisiae Bioassay

Three strains of Saccharomyces cerevisiae were used in the bioassay. Strain S288C, a haploid prototroph, was used to detect forward mutagenesis (Brusick 1970); Strain S211, a haploid methionine auxotroph was used to detect reverse mutagenesis. Diploid Strain D7 was used to detect mitotic recombination (reciprocal crossing-over) between ade 2 alleles, and nonreciprocal mitotic gene conversion involving the alleles try 5-12 and try 5-27 (Zimmermann et al., 1975). Recombination between ade 2 alleles results in red/pink sectors in the colonies. Strains S288C and S211 were obtained from Dr. David J. Brusick of Litton Bionetics. Strain D7 was obtained from Dr. C. Gairola of Tobacco and Health Institute, University of Kentucky. Diethylsulfate (80 µg/plate) and nitrosoguanidine (10 µg/plate) served as diagnostic mutagens for forward and reverse mutagenesis and gene conversion, and nitrosoguanidine (10 µg/plate) for mitotic crossover. Mutagens requiring metabolic activation, for example 2-aminofluorene (50 µg/plate), benzo(a)pyrene (25 µg/plate), aflatoxin B_1 (5 µg/ plate) could not be detected in the assay even in the presence of rat liver homogenate. Preincubation of the test organism with promutagens in the presence of S-9 Mix in 0.9% saline before plating on the agar medium (liquid suspension assay) also did not permit their detection. Difficulty in detecting mutagens requiring metabolic activation with this assay has also been noted by others (Simmons, 1979; Gairola, personal communication). Therefore, the assay was performed in the absence of mammalian metabolic activation. A plate incorporation assay was used with crude wastewater concentrates and separated fractions while a media water assay was performed on unconcentrated wastewater. The experimental details for studying different categories of genetic damage in yeast are given below.

Forward and Reverse Mutagenesis--

S-288C and S-211 yeast cells were grown overnight in yeast extract peptone dextrose broth (YEPD broth), centrifuged and resuspended in sterile saline. For forward mutagenesis in plate incorporation assays, 0.1 ml of the yeast S-288C cell suspension and appropriate quantity of the test concentrate were added to 3 ml of 0.75% soft agar which was overlaid on YEPD plates. Following 48 hours incubation at 30°C, the plates were replica plated onto yeast minimal medium supplemented with 30 µg/ml canavanine sulfate. Canavanine sulfate resistant colonies were counted after growth for five to six days at 30°C. For reverse mutagenesis with S211, the soft agar was overlaid on yeast minimal agar plates and methionine prototrophic revertants were scored after incubation for five to six days at 30°C.

Unconcentrated wastewaters were studied by making filter sterilized wastewater into YEPD agar for forward mutagenesis and into yeast minimal medium for reverse mutagenesis. The volume of wastewater tested was 16.5 ml/assay. The remaining procedure was followed as described above.

Mitotic Crossover and Gene Conversion--

Yeast strain D7 was grown overnight on YEPD broth. Cells were harvested and resuspended in sterile 0.85% saline. For mitotic crossover in plate incorporation assay, 0.1 ml of the appropriate dilution of the cells in saline and test wastewater concentrate were added to 3 ml molten top agar. The agar was mixed and overlaid on a YEPD plate. After five days incubation at 30°C, the plates were scored for red/ pink colonies. Cell suspension and wastewater concentrate mixed in molten top agar were overlaid on yeast minimal media supplemented with 20 µg/ml adenine sulfate for mitotic gene conversion. The plates were scored for tryptophan independent colonies after five days incubation at 30°C. YEPD agar was made in filter sterilized wastewater for studies with unconcentrated wastewaters. This enabled incorporation of up to 16.5 ml wastewater/assay.

Cell Transformation Assay Involving Baby Hamster Kidney Cells (BHK_{21} Cℓ 13)

Cultured mammalian cells have been used in recent years as an *in vitro* bioassay for carcinogenic compounds. Cultured mammalian cells have several advantages over other *in vitro* assays. First, mammalian cells are eukaryotic diploid cells which have a closer relationship to human carcinogenesis. Second, the observed change in culture consists of the loss of at least some of the growth control process. The loss of growth control is believed to be one of the stages in the progression from normal to malignant cells. Third, in many cases the cells transformed in culture will produce tumors when injected into animals. Fourth, cultured cells may be susceptible to epigenetic action of carcinogens as well as direct interactions with DNA.

In this study, the transformation assay of Purchase *et al.*, (1976) was employed using neonatal Syrian hamster kidney fibroblasts (BHK_{21} Cℓ 13). The assay has been reported to be 90% accurate in distinguishing between carcinogens and noncarcinogens (Styles, 1977). The procedure was followed as described by the authors with two modifications: (a) cells were treated with

the test material in monolayers to minimize the problem of cell damage and of aggregation which occurred during treatment of the cell suspensions, and (b) the concentration of agar in the base layer was increased to 0.5% in the soft agar assay. At the lower agar concentration recommended by Purchase et al., (1976); the gel was too thin and consequently cells settled to the bottom and started to grow as monolayers instead of forming discrete colonies. The cells were treated with the organic concentrates of wastewater in basal salts medium containing the Ames metabolic activation system (S-9 Mix), for 1 hour at 37°C. Following treatment, the test medium was removed and replaced with the standard growth medium with 10% serum. The cells were allowed to grow for 24 hours after which they were removed by trypsinization, and added to a 1.5 ml overlay of 0.3% agar in tissue culture medium. The agar overlay was poured over a base agar layer contained in a 60 ml petri dish. Transformation was quantized after 14 to 21 days incubation by counting the colonies on the soft agar. Nontransformed cells do not grow in soft agar while transformed cells divide and form colonies. Colonies on soft agar were counted after staining with the supravital stain neutral red. The stain is actively concentrated by living cells causing the colonies to appear bright ruby red against the light red background of the agar. Polyomavirus (PY1) - transformed BHK cells were utilized to check the performance of the soft agar assay. Diagnostic carcinogens used in the study were: benzo(a)pyrene, dimethylnitrosamine, and aflatoxin B_1.

The cell transformation assay could not be used in a routine manner because of the excessive time requirement for the assay and its erratic behavior. It was only used in selective instances to confirm the results obtained with the microbial assays.

SECTION 6

RESULTS AND DISCUSSION

GENERAL CONSIDERATIONS

Identification of every compound of concern in a complex uncharacterized medium such as municipal wastewater is an extremely difficult task. Even if identification could be accomplished, nothing would be revealed from the data with respect to any health effects linked to their presence in wastewaters except in a few cases where information is available in the literature. An alternative strategy is to use rapid in vitro bioassays for detecting presumptive biological activity in wastewater samples and locating active fractions, and then subjecting the active fraction to physicochemical analysis to fix molecular composition. This strategy eliminates the need for laborious analysis of samples that do not contain hazardous compounds, and enables detection of unknown biologically-active compounds. The present investigation identified mutagenic components of municipal wastewater and examined their fate during AWT treatment.

The analysis of mutagenicity of municipal wastewaters is complicated by many technical problems. Wastewaters, like any complex environmental sample, contain mutagenic substances in a matrix of innocuous and toxic substances. These substances may inhibit or modify the mutagenic response and restrict the sample volume that can be assayed. Consequently, a clear mutagenic response and a linear dose-response curve as one generally finds with pure compounds may not always be seen. The presence of many interfering materials in wastewater also presents a problem. For example, the amino acid histidine, which is present in municipal wastewater in small quantities (Faust and Hunter, 1971), interferes with the Ames' Salmonella assay by artifactually increasing the number of spontaneous revertants. Glutathione, a naturally occurring tripeptide, has been shown to modify the response of many mutagens in the Ames' Salmonella assay (Nemato et al., 1978); Rannug et al., 1978). The presence of glutathione and other sulfhydryl agents with similar action is very likely in municipal wastewaters because of their biological origin. A further problem is caused by the presence of mutagenic compounds in municipal wastewaters at low levels. This often necessitates preconcentration of compounds to meet the detection limit of the bioassay.

PERFORMANCE EVALUATION OF IN VITRO BIOASSAYS FOR DETECTION OF MUTAGENS/CARCINOGENS IN MUNICIPAL WASTEWATERS

In order to determine that the mutagens present in unconcentrated wastewaters or crude organic concentrates of wastewaters will be detected with the assay methods selected, we evaluated wastewater samples which were spiked

with known mutagens and carcinogens. Studies with unconcentrated waters were conducted using the secondary effluents from Lake George Village, NY. These wastewaters were repeatedly found to be free of mutagens and carcinogens in the Ames' assay and S. cerevisiae strain D3 and D4. Thus, complications arising due to background activity were expected to be minimal. Among the mutagens evaluated were those not requiring metabolic activation: nitrosoguanidine, bis(4chlorobutyl) ether, methylmethane sulfonate, and 9-aminoacridine; and those requiring metabolic activation: 2-aminofluorene, benzo(a)pyrene and aflatoxin B_1. The studies were confined to the Salmonella test which was the assay method most extensively used during the project.

The results from the plate incorporation assay on spiked wastewaters are shown in Table 5. The efficiency of detection of mutagens was found to be high with most mutagens. Nitrosoguanidine showed somewhat lower mutagenic activity in strain TA100, and bis(4-chlorobutyl)ether, methylmethanesulfonate and 9-aminoacridine in TA98. The lower mutagenic response was not due to detoxification or instability of the mutagens in wastewaters since they could be detected with 100% efficiency with other Salmonella tester strains. The results show that mutagens can be detected in wastewaters without significant interference from the components of wastewater or inactivation of the mammalian metabolic activation system.

TABLE 5. EFFICIENCY OF DETECTION OF MUTAGENS IN MUNICIPAL WASTEWATERS

Mutagens Tested	Concentration per Plate (µg)	Metabolic Activation	Efficiency of Detection (%)*		
			TA98	TA1000	TA1537
Nitrosoguanidine	2	-	135	79	-
Bis(4-chlorobutyl)ether	100	-	59	112	-
Methylmethanesulfonate	50	-	62	95	100
9-Aminoacridine	10	-	48	86	128
2-Aminofluorene	10	+	100	100	76
Benzo(a)pyrene	5	+	102	101	112
Aflatoxin B_1	1	+	95	133	-

*$\frac{\text{Number of revertants in spiked secondary effluent}}{\text{Number of revertants in spiked distilled water}} \times 100$

In view of the possibility that interfering and/or masking agents may be concentrated during recovery of organics from wastewater, the efficacy of the Ames Salmonella assay for detection of mutagens when present in crude organic concentrates of wastewater was also examined. In these studies, the organic mixtures recovered from Piscataway wastewaters by various concentration methods were spiked with known mutagens and subjected to the conventional Ames assay (spot test and plate incorporation assay). The mutagens tested in spot test were nitrosoguanidine (2 µg/plate) and 2-aminofluorene (10 µg/plate). In the presence of the wastewater concentrate equivalent to 50 ml wastewater, no interference in the formation of a ring

around the mutagen spot was noted. Plate incorporation studies with wastewater concentrates spiked with various concentrations of benzo(a)pyrene further revealed the absence of any interfering or masking substance in the concentrates (Table 6).

TABLE 6. MUTAGENICITY OF WASTEWATER CONCENTRATES SPIKED WITH BENZO(a)PYRENE

Salmonella Tester Strain	Number of Revertants (+S9)			
	Spontaneous	Organic Concentrate Alone	Wastewater Concentrates Spiked with Benzo(a)pyrene*	
			1 µg BaP/plate	5 µg BaP/plate
SECONDARY EFFLUENT				
TA98	85	92	314	500
TA100	234	254	500	1000
AWT MIDPOINT				
TA98	85	127	269	500
TA100	234	254	500	1000
AWT FINAL				
TA98	85	103	215	500
TA100	234	252	500	1000

*The organic concentrate (equivalent to 50 ml wastewater) prepared from Piscataway wastewaters with polyurethane foam plugs were spiked with various concentrations of benzo(a)pyrene and subjected to the Ames *Salmonella* plate incorporation assay.

Although the limited studies conducted with spiked concentrates revealed that mutagens in wastewater concentrates would be detected by spot test and plate incorporation, this did not appear to be the case with all mutagens. This conclusion was reached based on the finding that the Piscataway wastewater concentrates which were nonmutagenic (without spiking) in the spot test and plate incorporation assay exhibited significant mutagenesis in the liquid suspension assay. (See Figures 7 and 8.) In view of this, the liquid suspension assay was chosen for assessment of mutagenic potential of wastewater concentrates and separated fractions over the plate incorporation assay and spot test.

EVALUATION OF PHYSICAL-CHEMICAL TREATMENT AT PISCATAWAY, MD.

In view of the generally recognized quality fluctuations in wastewaters, two samples collected several weeks apart from the Piscataway AWT plant were tested. For the studies with unconcentrated wastewaters and crude organic concentrates, samples were collected on different dates.

Mutagenic Response of Unconcentrated Wastewaters

Secondary, as well as AWT effluent, showed mutagenesis in the base pair substitution mutants TA100 and TA1535 of Salmonella typhimurium (See Figures 9 and 10). The mutagenic response increased with increase in wastewater concentration in the assay but was not linear with dose. The mutagens responsible for the effect were inactivated to a large extent by the presence of mammalian liver enzymes in the assay. The microsomal enzyme system used in the Ames assay consists of an array of enzymes which detoxify xenobiotic compounds in the liver. It has been demonstrated with other compounds that this enzyme system has the capability of inactivating direct-acting mutagens as well as activating promutagens (Won et al., 1976). The wastewaters failed to revert the frameshift mutant TA98. The inability to detect frameshift mutagens may be linked to either no such mutagens present or to the high sensitivity of this strain towards toxicants in the wastewater. However, decreasing the volume of wastewater in the assay did not induce a mutagenic response.

Chemical mutagens that enter the human body through the diet, or in other ways, are often excreted as water soluble conjugates. This is a detoxification process, and the conjugates are biologically inactive. Thus, the possibility exists that some mutagens will be present in municipal wastewaters as conjugates, such as glucuronides, which may later liberate the mutagen. Since glucuronides are not split by the enzymes in Salmonella or in the rat liver homogenate, glucuronidase was incorporated in the assay to cause hydrolysis (Yamasaki and Ames, 1977; and Commoner et al., 1974). The presence of glucuronidase in the assay did not result in an increased mutagenicity of the wastewater samples studied. This demonstrated that any mutagens present were not conjugated as glucuronides within limits of detectability.

The Ames assay mutagenicity data for the Piscataway AWT plant was especially interesting because of the inclusion of a sample at a midpoint in the AWT processes. A comparison of the mutagenic response of the AWT influent and midpoint effluent showed that lime addition and/or recarbonation were capable of promoting a mutagenic response. Some of these compounds were direct acting, whereas others required metabolic activation. At the Piscataway AWT plant, recarbonation was performed with liquified carbon dioxide, not with flue gas from the sludge incinerator.

Thus, the increased mutagenicity was not due to contamination of the wastewater with the combustion products. The possible mechanisms for increase in mutagenesis of wastewaters after liming/recarbonation are: (a) mutagens may have been synthesized under the alkaline conditions of liming or during recarbonation from precursors present in the wastewaters; (b) mutagenic compounds may have been added to the wastewaters as contaminants of the chemical additives used in the liming/recarbonation steps; (c) liming and recarbonation process may be responsible for removing some masking agents or toxicants which could have prevented expression of activity in the secondary effluent. With the available data, none of the three possibilities can be verified.

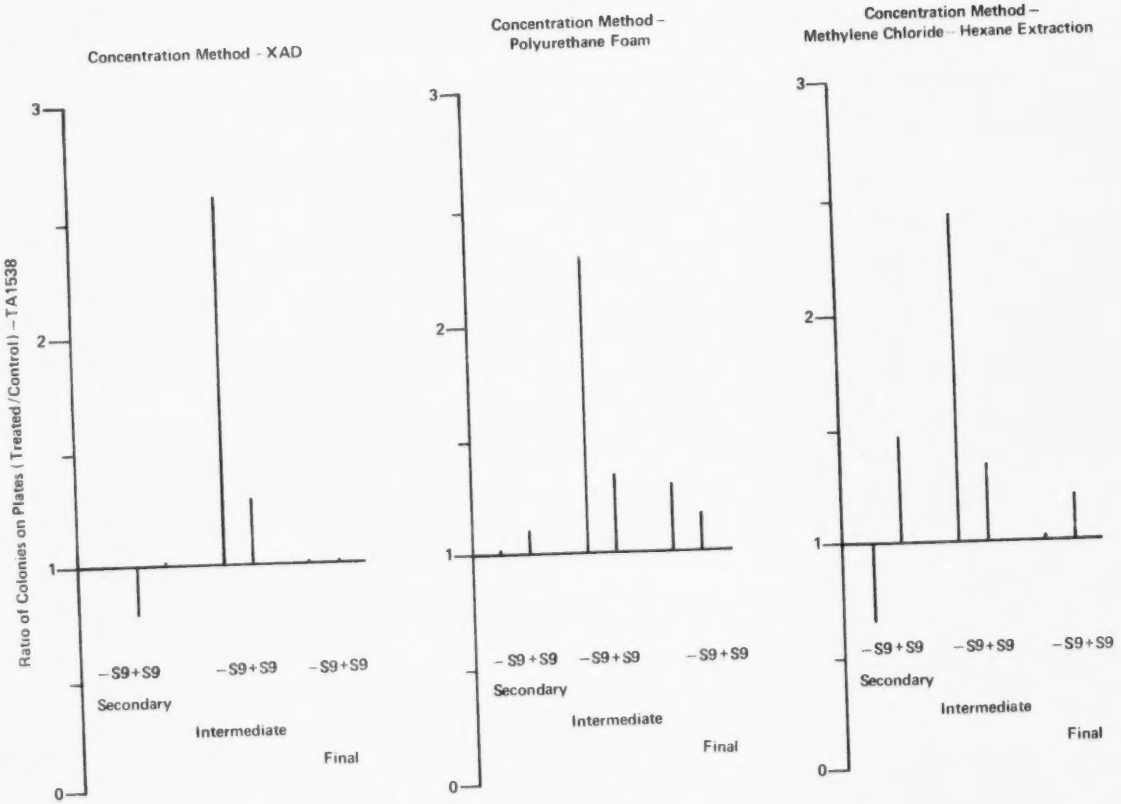


Figure 7. Mutagenic response of the organic mixtures recovered from secondary effluent, and effluent after partial and full AWT at Piscataway (sample 1) with Salmonella strain TA1538

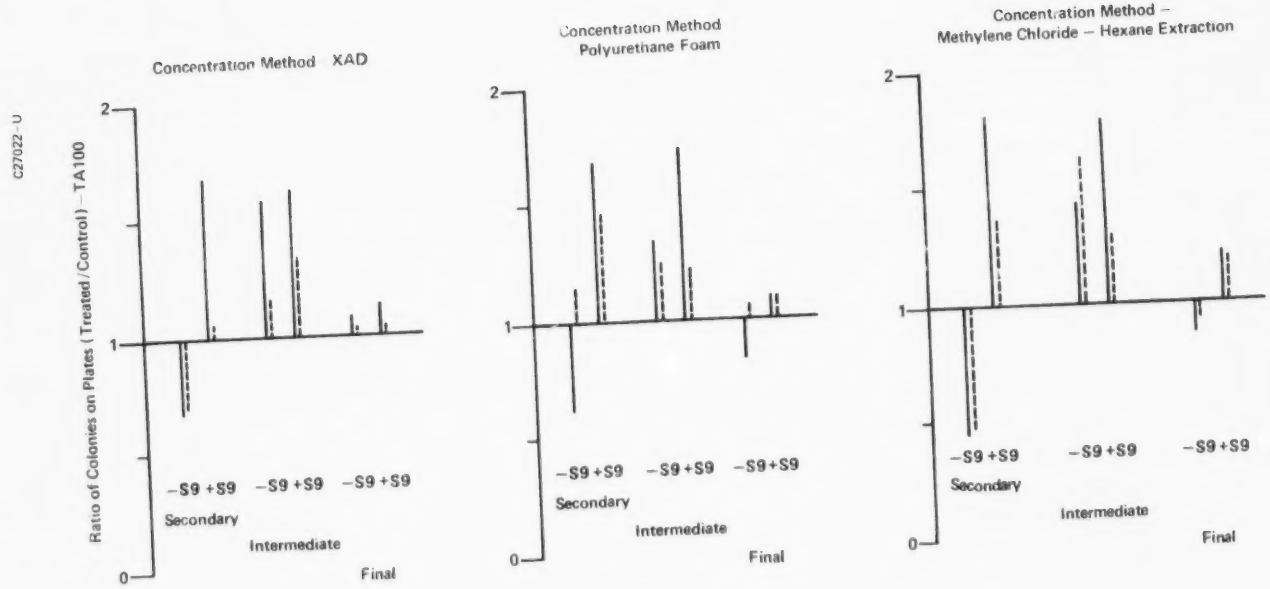


Figure 8. Mutagenic response of the organic mixtures recovered from secondary municipal effluent after partial and full AWT at Piscataway (sample 1) with Salmonella strain TA100

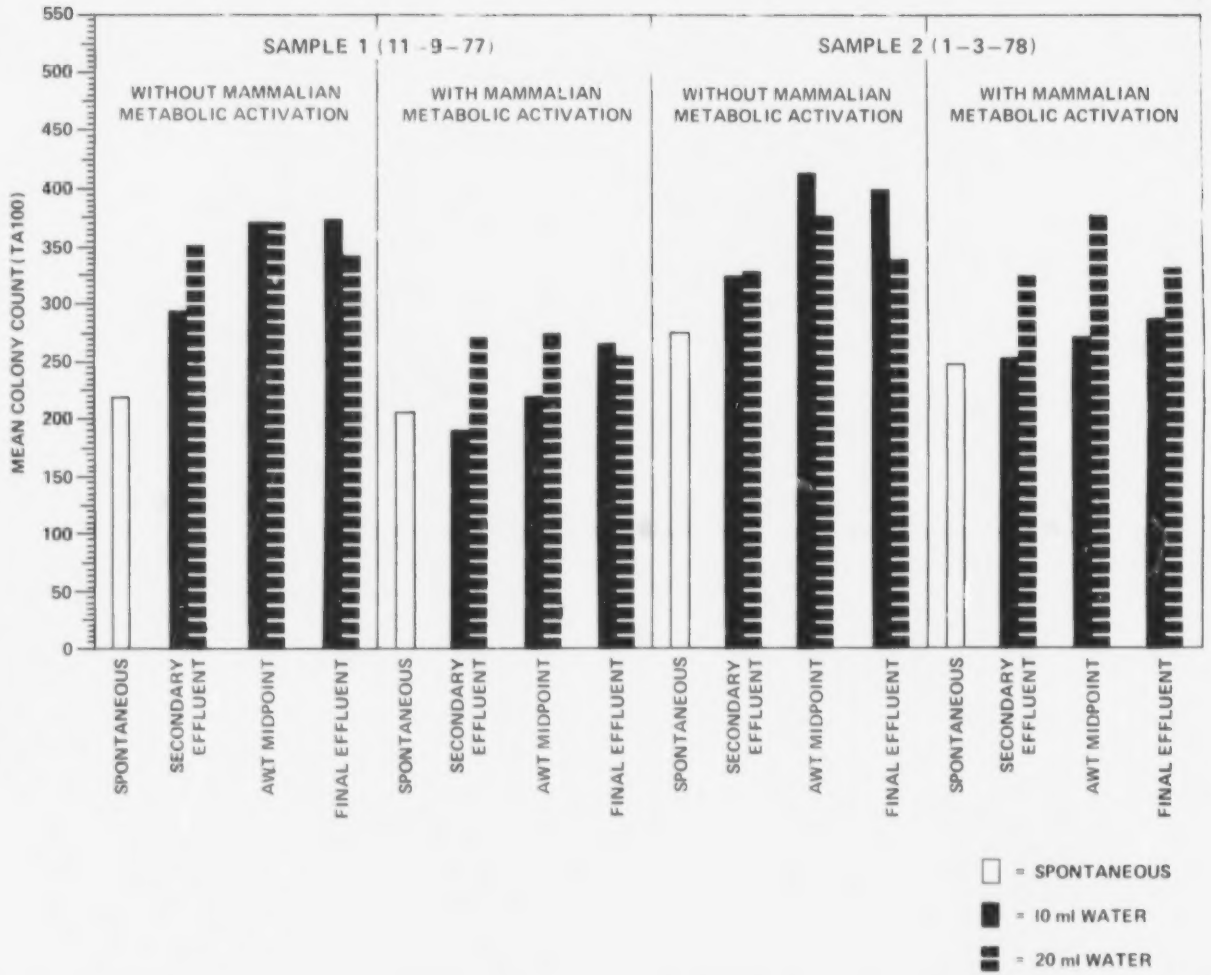


Figure 9. Mutagenic response of the unconcentrated wastewaters from Piscataway AWT plant, with Salmonella strain TA100

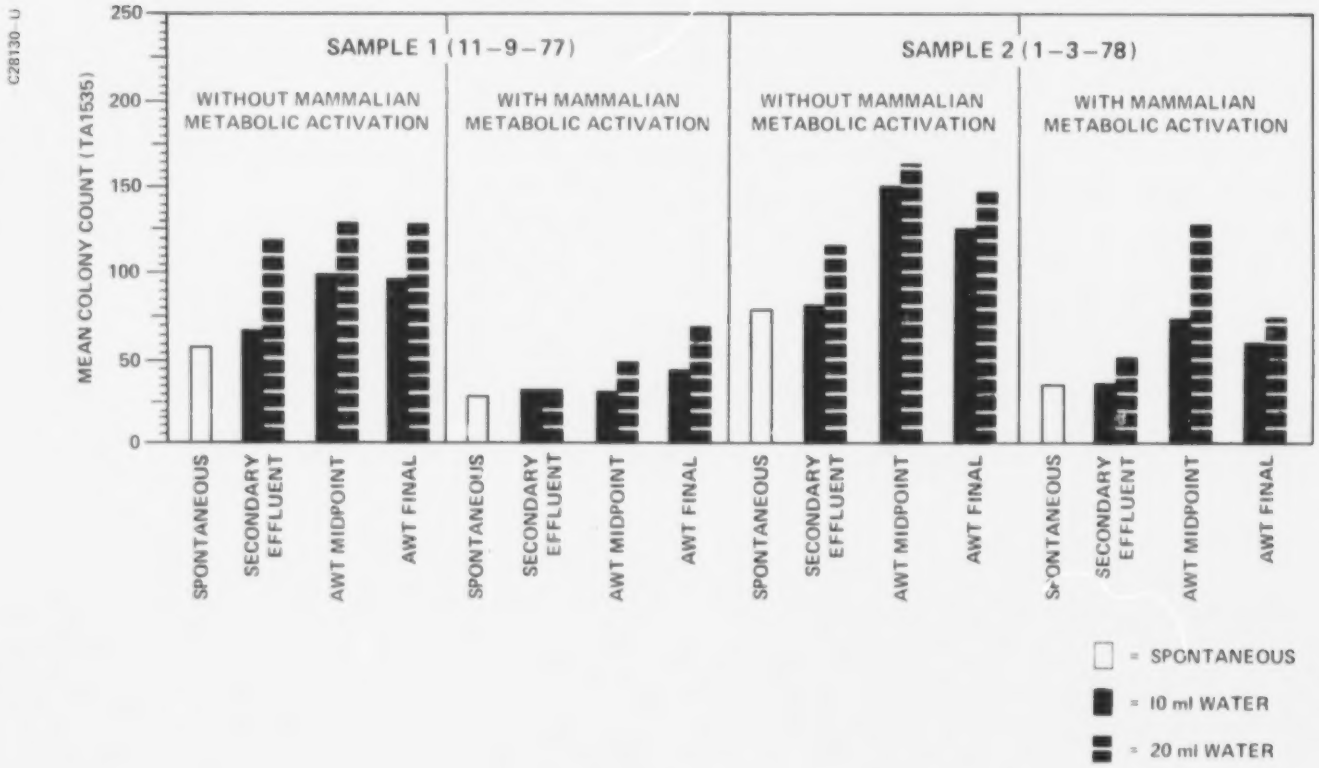


Figure 10. Mutagenic response of the unconcentrated wastewaters from Piscataway AWT plant, with Salmonella strain TA1535

The increased mutagenicity observed at the midpoint collection site was partially removed during breakpoint chlorination and/or carbon filtration. However, the overall mutagenic response observed in the final effluent was no less than that present in the influent wastewaters. It was not clear from this study whether the mutagens detected in the final effluent were originally present in the wastewaters and were refractory to the AWT process, or whether they were added during treatment. However, the levels of mutagenic activity detected in unconcentrated wastewaters were very low, and the difference between the activity in the influent, midpoint, and final effluent samples was not great. This low level of activity made the interpretation of these results difficult. Although the above interpretation is suggested from the results, further experiments, in greater depth will be needed to show conclusively that the mutagenic activity is increased after liming/recarbonation.

The unconcentrated wastewaters from Piscataway showed no genetic activity in media water assays using yeast strains S211 or D-7. A weak mutagenic response was noted in forward mutagenesis in the presence of AWT midpoint and AWT final effluents. Such increase was absent in the secondary effluent samples (Table 7). The results from mitotic gene conversion with strain D-7 were inconclusive.

TABLE 7. GENETIC ACTIVITY OF PISCATAWAY UNCONCENTRATED WASTEWATERS IN S. CEREVISIAE (MEDIA WATER ASSAY)

Genetic Event	Tester Strain	Spontaneous Mutants/ Revertants/ Recombinants	Positive Control (Nitro-soguani-dine)	Genetic Activity in the Presence of 16.5 ml Wastewater/Assay		
				Secondary Effluent	AWT Midpoint	AWT Final
Forward Mutagenesis	S288C	133	261*	137	162	179
Reverse Mutagenesis	S211	11	48	15	12	10
Mitotic Crossover	D-7	1	5	1	1	1

*Lower than usual reversion rate is probably due to breakdown of nitrosoguanidine solution.

The weak mutagenic response noted with the yeast assay was not unexpected since the assay is known to be several orders of magnitude less sensitive than the Ames Salmonella assay. For example, the diagnostic mutagen dimethylnitrosoamine has been reported to cause mutagenesis in Salmonella at a dose 100 times lower than necessary for Saccharomyces strain D-3 (Brusick and Mayer, 1973).

Recovery of Organics from Wastewater and Bioassay of the Crude Concentrates

Recovery of Organics--

The total organic carbon content of the wastewaters and the proportion

recovered by the various concentration methods is shown in Table 8.

TABLE 8. RECOVERY OF ORGANIC COMPOUNDS FROM PISCATAWAY WASTEWATERS BY DIFFERENT COLLECTION METHODS (SAMPLE 1; DATE 3-27-78)

Sample	Total Organic Carbon (ppm)	Percent Carbon Recovered By		
		Foam	XAD	Methylene Chloride-Hexane Extraction
Secondary Effluent	12.2	2	9.8	24.6
Intermediate Effluent	2.8	8.9	13.6	7.8
Tertiary Effluent	1.4	6.8	46.0	10.0

The measured levels of organic carbon in the wastewaters were surprisingly low. The samples for TOC analysis were taken after filtration through glass wool and hence the lower TOC levels may be due to removal of particulate organic carbon. The secondary effluent contained 12.2 ppm organic carbon. The midpoint sample represented a 77% reduction in carbon, and the carbon level in AWT effluent was reduced by 88%. The percent of carbon recovered by the collection methods varied with the level of carbon in the wastewaters. Whereas, in the case of foam and XAD, the retention efficiency increased with decreasing carbon in wastewater, the reverse was true with solvent extraction. On the basis of carbon recovery, solvent extraction gave the best results with secondary effluent, while XAD was best with intermediate and tertiary effluents.

Mutagenic Response With Crude Organic Mixtures

Ames' *Salmonella* assay--

Mutagenic responses in *Salmonella* strains TA100 and TA1538 of the recovered organics from Piscataway samples (date 3-27-78) are shown in Figures 7 and 8. Significant mutagenesis was not observed in strains TA1535, TA98 and TA1537. Although polyurethane foam collected the least amount of organic carbon, the recovered carbon possessed mutagenic activity roughly equivalent to that recovered with XAD and solvent suggesting some specificity of foam plugs for recovering mutagens. The organic mixture recovered from AWT influent was mutagenic for *Salmonella* strain TA100 but not to a significant extent in TA1538. Following liming and/or recarbonation of the influent, the mutagenic response shown by TA100 remained unaffected but there was a significant increase in mutagenicity with TA1538. The findings show that liming and/or recarbonation processes have the potential of introducing compounds capable of causing frameshift mutation. The concentration methods employed failed to recover base pair substitution mutagens shown to be present in the unconcentrated intermediate effluent (Figures 9 and 10).

The final AWT effluent did not exhibit significant mutagenic activity in any of the *Salmonella* strains tested. This does not show that the mutagens

present in influent wastewaters and those introduced during liming and/or recarbonation were effectively removed during the final stage of treatment, since mutagens were detected in unconcentrated samples of AWT effluents. The inability of the concentration methods to recover these mutagenic substances from AWT effluent appears to be responsible for their absence. As the mutagens in unconcentrated wastewaters were detected by preparing the bottom agar with the test wastewater, diffusion of mutagens from the bottom layer to the top layer was necessary. Since the indicator organism and S-9 mix were added to the top agar layer, only in this way could mutagens be acted on by liver enzymes, or cause reversion in S. typhimurium. Our studies have shown that nonpolar compounds such as benzo(a)pyrene which fail to diffuse in aqueous medium are not detected with high efficiency under these conditions. This may indicate that the mutagens detected in unconcentrated wastewater were polar and, therefore, probably not efficiently recovered by adsorption on XAD or foam or by solvent extraction. On the other hand, those mutagens present in influent wastewaters which could be recovered by the above concentration methods were efficiently removed during treatment. The inability of the concentration methods, employed in this study, to recover mutagens of a polar nature combined with the efficient removal of the nonpolar mutagens by the treatment processes appear to be responsible for the absence of mutagenic activity in the organic mixture recovered from AWT effluent.

The crude concentrates obtained from the second samples of Piscataway (date 5-10-78) were also mutagenic but to a lower extent than concentrates from the first sample (Figure 11). The activity increased after the liming recarbonation step as reported with the earlier sample.

Yeast Mutagenesis Assay--

The crude concentrates from Piscataway wastewaters collected on 3-27-78 failed to induce reverse mutagenesis or mitotic recombination in yeast. In the forward mutagenicity assay in Saccharomyces strain S288C, the wastewaters caused a small increase in the number of canavanine sulfate resistant colonies (Table 9). The mutagenic response followed the general trends observed with the Ames' assay, that is, the intermediate effluent was

TABLE 9. GENETIC ACTIVITY OF PISCATAWAY WASTEWATER CONCENTRATES IN S. CEREVISIAE FORWARD MUTAGENESIS ASSAY (STRAIN S288C) EQUIVALENT TO 100 ml WASTEWATER

	Sample 1 (3-27-78)	Sample 2 (5-10-78)
Spontaneous Mutation	36	62
Positive Control (Nitrosoguanidine)	>1000	>500
Number of Canavanine Sulfate Resistant Mutants in the Presence of Concentrations*		
XAD Resin		
Secondary Effluent	61	72
AWT Midpoint	81	79
AWT Final	58	57

Polyurethane Foam		
Secondary Effluent	49	77
AWT Midpoint	63	100
AWT Final	67	49
Solvent Extract		
Secondary Effluent	99	88
AWT Midpoint	88	56
AWT Final	65	28

*Concentrate equivalent to 100 ml wastewater/assay. No S-9 added.

slightly more mutagenic than either the secondary or final effluent. The second sample collected from Piscataway (5-10-78) showed little mutagenic activity, although the Ames assay for this sample was positive (Figure 11). This was indicative of the low sensitivity of the yeast assay.

Mammalian Cell Transformation Assay--

Several problems were encountered with the cell transformation assay. Although positive results could be obtained with many known carcinogens such as aflatoxin B_1, dimethylnitrosoamine, and benzo(a)pyrene, the system proved to be highly erratic. At times, dose-dependent colony formation was obtained with the known carcinogens, while at other times the same concentration of these known carcinogens would cause extensive cell death, or the spontaneous colony number would rise to an unacceptable level.

Many parameters caused significant variations in this assay. One of the most striking was the effect of the S-9 mix, which was extremely toxic to the cells. This was shown by the complete inhibition of cell growth after a four-hour incubation in the presence of S-9. On microscopic examination of the cells in soft agar, the cell membrane appeared ragged and noncontinuous. Shorter incubation times with S-9 mix alleviated some of the overt toxicity; however, extensive cell damage may still have been present in some experiments to cause the negative results.

The process of transformation is a multistep procedure, and the BHK cells have progressed through part of the first stage. The ability of these cells to grow in soft agar is governed by many constituents of the media, including cation concentration, in addition to the transforming property of the test compound. It was believed that some media constituent was not constant from experiment to experiment, and this was causing the sporatic appearance of numerous colonies on the control plates. Although investigation of the cause of sporatic growth on the control plates was never carried out, it appeared to correspond with the use of different batches of calf serum.

One preliminary experiment was performed with the crude organic mixtures recovered from Piscataway wastewaters by XAD resin and polyurethane foam plugs. A variety of concentrations of the organic mixture representing a range of 1 to 200 ml wastewater were tested. The results showed an increased number of colonies with samples obtained from secondary and AWT midpoint samples. The organics recovered from the final effluent elicited at best

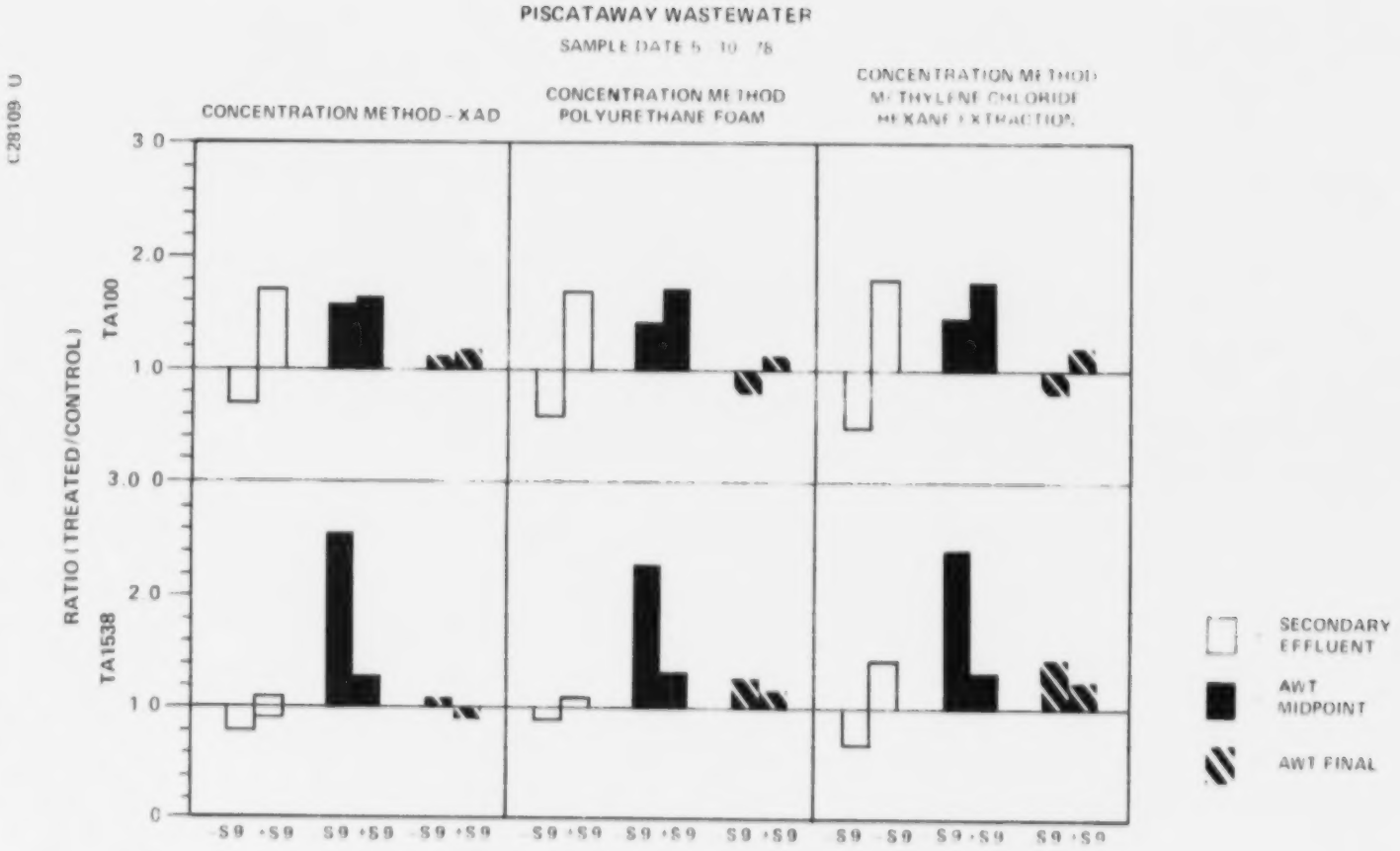


Figure 11. Mutagenic response of the organic mixtures recovered from secondary effluent and effluent after partial and full AWT (sample 2) with Salmonella strains TA1538 and TA100

only a marginal increase in the number of colonies. It should be of interest to note that the Ames' plate incorporation assay on the Piscataway concentrates gave negative results, and at best a doubling of colonies was observed with wastewater concentrates in the liquid suspension assay.

Chemical Separation of Crude Organic Concentrates and Bioassay of Separated Fractions

The crude organic concentrates of wastewaters were chemically fractionated and each fraction evaluated for mutagenic activity. The objectives of the study were: (a) to uncover the activity which may have been masked because of the complexity of the crude concentrates; and (b) to identify the chemical class(es) responsible for the activity. Because of the large number of separated fractions involved, it was not possible to test mutagenicity of each fraction using all three bioassay procedures. The Ames' Salmonella assay was selected for these studies, because, of the three assay methods, it has the advantage of speed, ease of testing, and generation of fairly precise results. In some studies, the yeast mutagenesis assay was also used.

Chemical Separation of Crude Concentrates--

Mass distribution of the crude organic mixture recovered from Piscataway wastewaters by XAD and polyurethane foam is shown in Table 10. TOC measurements on the separated fractions from solvent extract could not be made because of limitation on the quantity available. It should be mentioned that the TOC of the separated fractions was greater than that of the crude concentrates. With some crude concentrates, the increase was as much as several-fold. In view of this, caution should be exercised in deriving conclusions from the absolute numbers. It is suggested that the data be used only in a qualitative manner to derive trends of the carbon distribution. The mechanism by which organic carbon was introduced during or after fractionation is unclear at this time. A blank fractionation run (without wastewater concentrate) revealed that the solvents and solutions used in the separation scheme were not responsible for the additional carbon. It appears that the separated fractions may have become concentrated as a result of solvent loss during storage.

The neutral fraction accounted for a major portion of the total carbon in the concentrates. This was found to be the case with both polyurethane foam and XAD concentrates. The concentration methods recovered various chemical classes with widely differing efficiencies. For example, whereas XAD resin effectively concentrated water soluble and ether insoluble compounds polyurethane foam recovered neutral and amphoteric compounds with greater efficiency. The efficiency of recovery of foam for neutral compounds was particularly high with tertiary effluent where the carbon content was low.

The organic distribution following liming/recarbonation steps revealed an increase in the amount of carbon in neutral (aliphatic, aromatic, and oxygenated), basic, and weak acid fractions. Breakpoint chlorination/carbon adsorption caused a decrease in the level of carbon in aliphatic and oxygenated neutrals and in basic fractions, but the carbon content of aromatic

TABLE 10. MASS DISTRIBUTION OF THE ORGANIC CARBON RECOVERED BY POLYURETHAN FOAM AND XAD RESIN FROM PISCATAWAY WASTEWATERS

Sample	Concentration Method	TOC in Separated Fractions (ppm)								
		Strong Acids	Weak Acids	Basic	Water Soluble	Ether Insoluble	Amphoteric	Aliphatic Neutrals	Aromatic Neutrals	Oxygenated Neutrals
Secondary Effluent	Polyurethane Foam	0.21	0.03	0.009	0.05	0.1	0.65	0.06	0.18	0.16
	XAD Resin	0.06	0.02	0.0007	1.84	0.68	0	0.12	0.1	0.44
AWT Midpoint	Polyurethane Foam	0.06	0.26	0.13	0.04	0.06	0.03	0.43	0.46	0.48
	XAD Resin	0.06	0.20	0	0.07	0.32	0	0.1	0.20	0.69
AWT Final	Polyurethane Foam	0.006	0.04	0	0.016	0.005	0	0.05	0.25	0.14
	XAD Resin	0.02	0.16	0.01	0.15	0.28	0.005	0.007	0.005	0.16

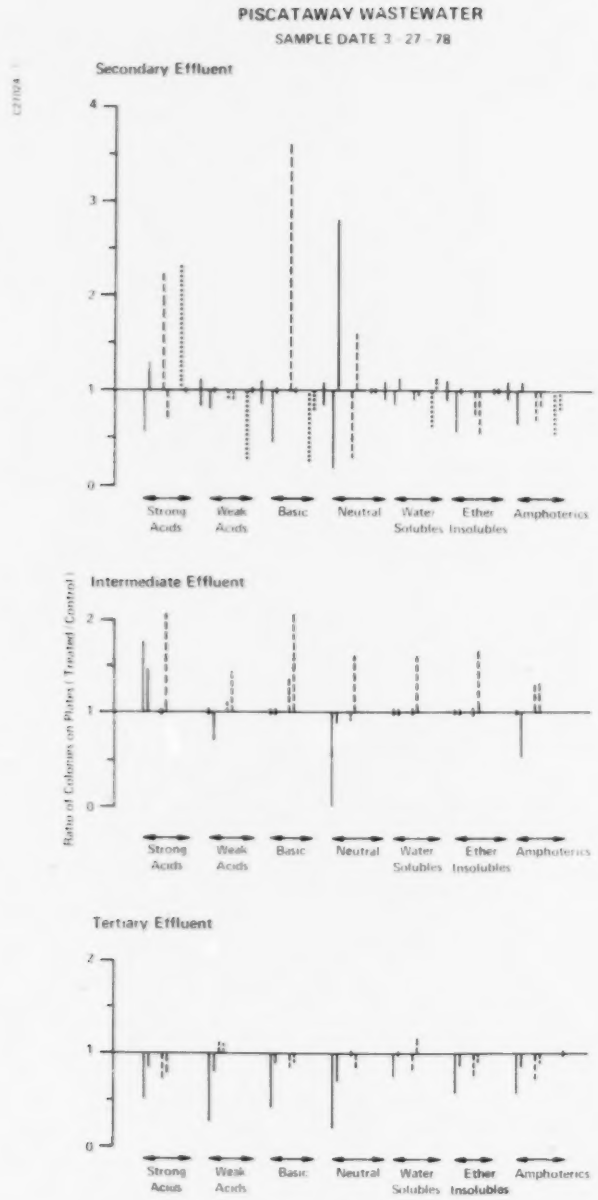
neutrals and weak acids remained high in the final effluent. Examination of the mass distribution data for other classes of chemicals revealed that strong acids, water soluble compounds, and ether insoluble compounds were partially removed during the treatment, while amphoteric compounds were completely removed.

Bioassay of Separated Fractions--

Ames Salmonella assay--The assay was performed on the separated fractions obtained from the crude concentrates from two Piscataway samples (dates 3-27-78 and 5-10-78). All the separated fractions from the first sample were tested in the Ames' assay initially at one concentration (fraction volume representing 100 ml wastewater). The fractions which showed mutagenic response were further tested in a dose-response manner. This testing approach was discontinued in the latter studies in favor of testing at least three concentrations for each separated fraction. The second approach proved to be less time consuming and also reduced the amount of material needed for testing.

The results from the Salmonella mutagenicity assay of the separated fractions for the 3-27-79 sample are shown in Figures 12 through 14. It was noted that the combined activity of all the fractions was generally greater than that of the crude concentrates. This increase could be due to two phenomena acting jointly or individually in the assay. First, toxicants may have been separated from mutagens allowing complete expression of the mutagens in the assay. Secondly, compounds which mask the activity of mutagens may have been separated into different fractions, away from the mutagens. The phenomenon of masking activity of known mutagens in complex mixtures has been reported in the literature (Shahin and Fournier, 1978). The masking action is not clearly understood, particularly for mutagens that do not require metabolic activation.

In terms of the quantities and classes of mutagenic substances recovered by the concentration procedures employed, the performance of polyurethane foam plugs was best; solvent extraction was second best; and XAD was poorest. The mutagenic activity recovered by polyurethane foam from AWT influent was distributed largely in basic, strong acid, and neutral fractions. In addition, the weak acid fraction was shown to be mutagenic when tests were performed at higher doses (Figure 15). Mutagenic response with these fractions was noted using Salmonella strains TA100 and TA98 without the presence of mammalian activation. Liming and recarbonation steps, while causing a partial decrease in the mutagenicity of the active fractions observed for the influent, resulted in the appearance of activity in certain new fractions. For example, ether insoluble and water soluble fractions of polyurethane foam concentrate of AWT influent were nonmutagenic, similar fractions representing treated effluent after liming and recarbonation were weakly to moderately mutagenic. Similarly, the amphoteric fraction of the solvent concentrate became mutagenic after liming and recarbonation (Figure 17). This provided further support to the earlier observation that liming and/or recarbonation processes introduced mutagens. It should be reiterated that recarbonation was not performed with the use of flue gas, and hence these mutagens were not combustion products. Similar increase in the number of mutagenic fractions was not noted with the



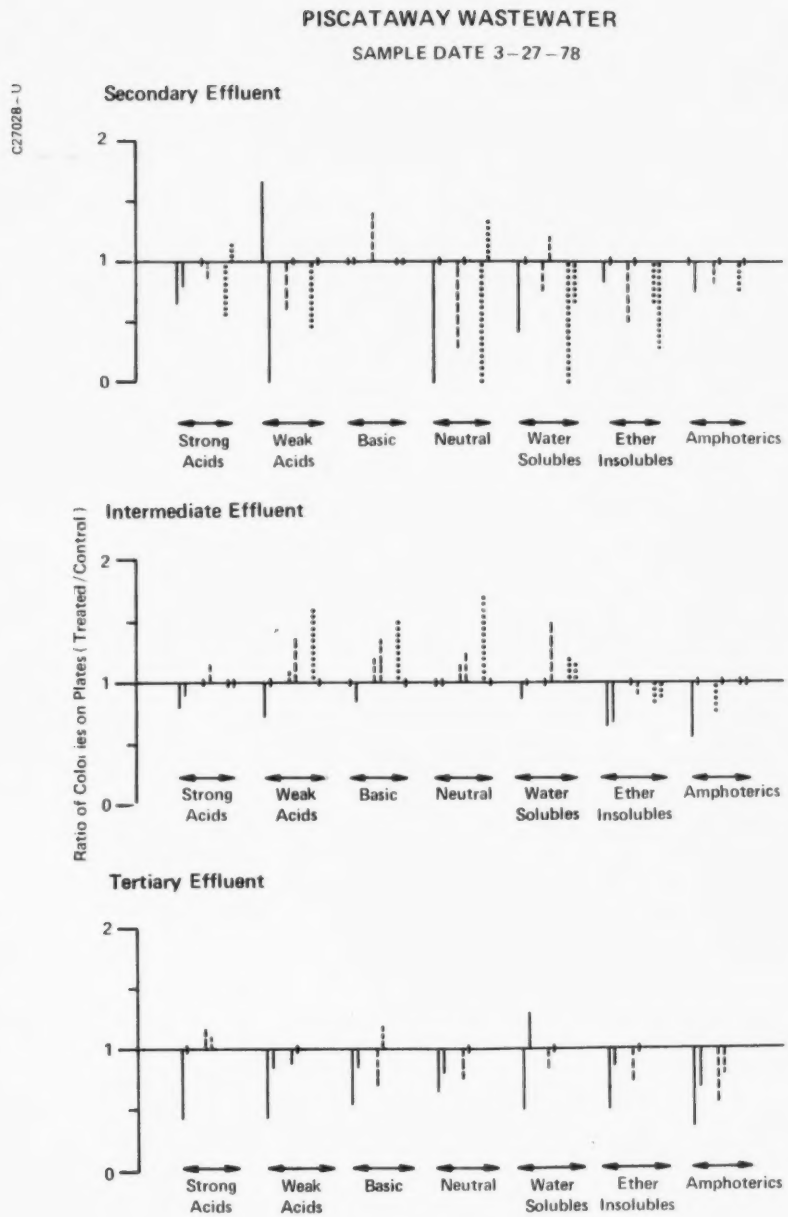
——— Salmonella Strain TA98

----- Salmonella Strain TA100

••••• Salmonella Strain TA1538

The response shown is for each fraction volume representing 100 ml wastewater. The first vertical line with each strain represents mutagenic activity in the absence of mammalian activation, the second vertical line is in the presence.

Figure 12. Mutagenic activity of the major classes of compounds recovered from Piscataway wastewaters with polyurethane foam plugs as sorbent (sample 1)



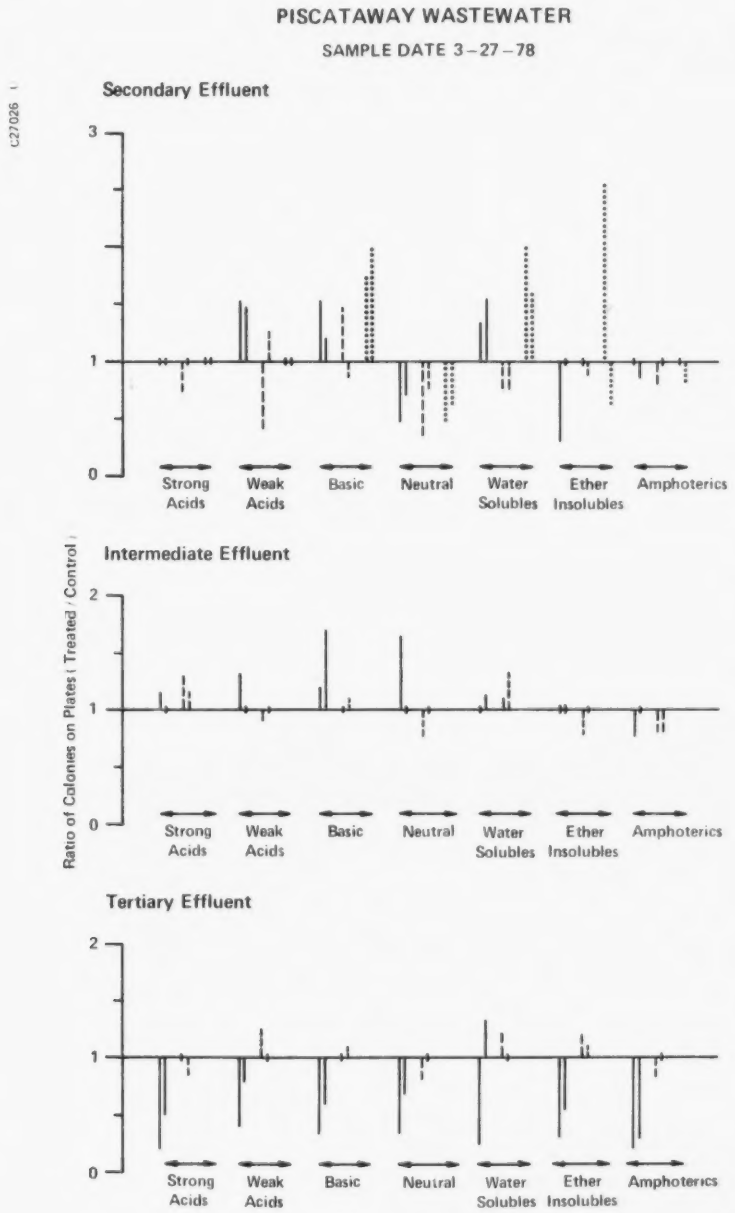
——— Salmonella Strain TA98

----- Salmonella Strain TA100

●●●●● Salmonella Strain TA1538

The first vertical line for each strain represents mutagenic activity in the absence of mammalian activation system, the second line is the presence. The response shown is for each fraction volume representing 100 ml wastewater. Data not shown for strain TA1538 for intermediate and teriary effluent.

Figure 13. Mutagenic activity of the major classes of compounds recovered from Piscataway wastewaters with XAD as sorbent (sample 1)



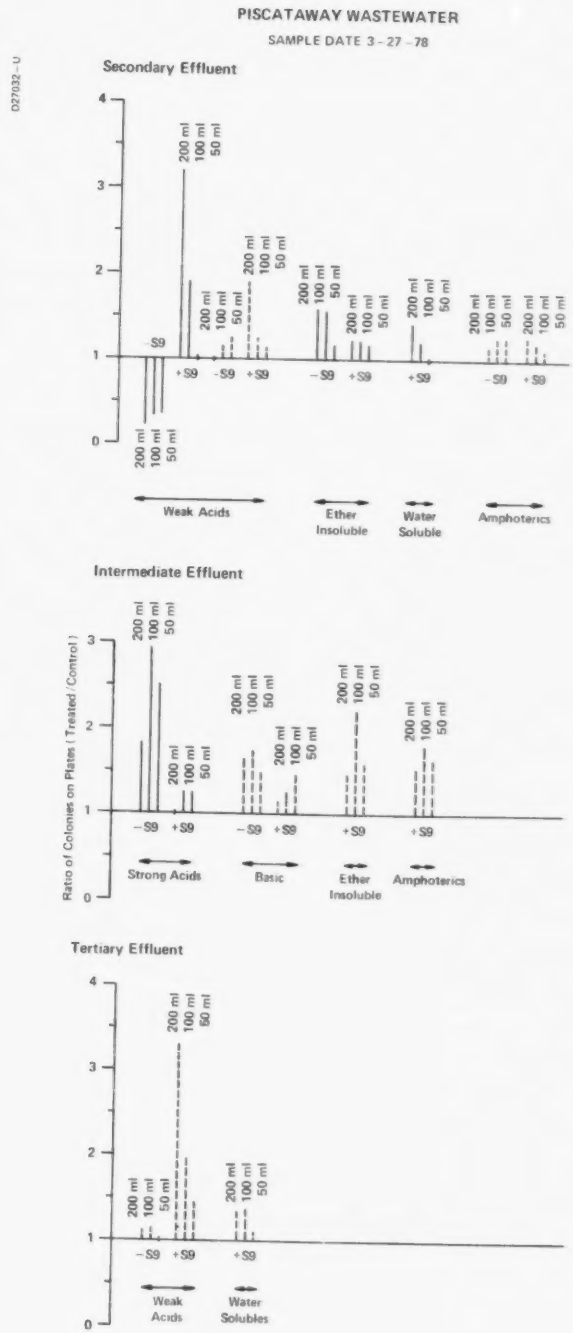
——— Salmonella Strain TA98

– – – – – Salmonella Strain TA100

• • • • • Salmonella Strain TA1538

The first vertical line with each strain represents mutagenic activity in the absence of mammalian activation system; the second line is in the presence. The response shown is for each fraction volume representing 100 ml wastewater. Data not shown for strain TA1538 for intermediate and tertiary effluent.

Figure 14. Mutagenic activity of the major classes of compounds recovered from Piscataway wastewaters by methylene chloride-hexane extraction (sample 1)



——— Salmonella Strain TA98
----- Salmonella Strain TA100

Figure 15. Dose-related response of the selected separate fractions of polyurethane foam concentrates of Piscataway wastewaters (sample 2)

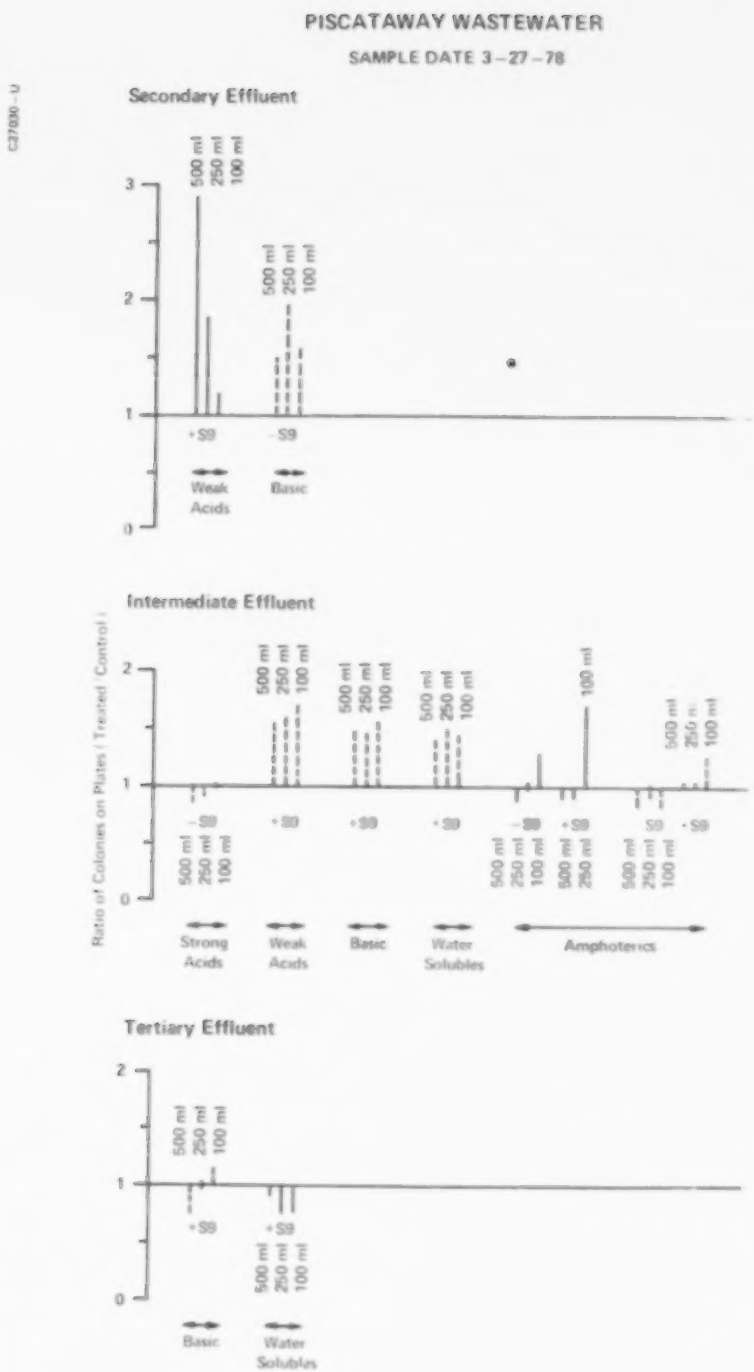
activity recovered by XAD sorption. Although the organic carbon content of many separated fractions was also increased after liming/recarbonation (Table 10), there was no direct correlation between the increased mutagenic activity and carbon content.

The separated fractions of the AWT effluent were generally very toxic to the Salmonella (Figures 12 through 16). By varying the concentration of the test fraction in the assay, mutagenic response became visible in the weak acid fraction but not in others (Figures 15 through 17). All three concentration methods used were equally effective in recovering the components of the effluent responsible for toxicity. These toxicants were not present in the midpoint effluent, and, therefore, their addition probably occurred during breakpoint chlorination and/or carbon filtration process.

Neutral fractions recovered by polyurethane foam, XAD, or solvent extraction were generally toxic to the Salmonella tester strains and, therefore, were subfractionated into aliphatic, aromatic, and oxygenated fractions prior to bioassay. The assays were performed with the concentration of recovered organics equivalent to 50 to 200 ml for the aliphatic and aromatic fractions and 10 to 50 ml for the more toxic oxygenated fraction. The positive mutagenic response by various subfractions of neutral components of wastewater was demonstrated largely in Salmonella strain TA98 in the absence of rat liver homogenate (Figures 18, 19, and 20). Nearly all the mutagenic activity for the neutral fraction of AWT influent was located in the oxygenated fraction. Efficient recovery of these compounds was achieved by solvent extraction. XAD adsorption partially recovered these compounds, but polyurethane foam failed to recover them. Oxygenated mutagenic compounds were also detected in the midpoint sample; however, these appeared to be of a different chemical nature, as they could be recovered by polyurethane foam. Furthermore, XAD adsorption and solvent extraction proved ineffective in recovering these mutagens. The data can be explained by suggesting that liming and/or recarbonation processes caused chemical modification of the oxygenated neutrals present in AWT influent. The altered compounds retained mutagenic activity but became resistant to recovery by XAD adsorption or solvent extraction. Alternatively, chemical changes in the wastewaters (for example, ionic strength or TOC) resulting from liming/recarbonation may have affected the adsorption properties of the neutral oxygenated fraction.

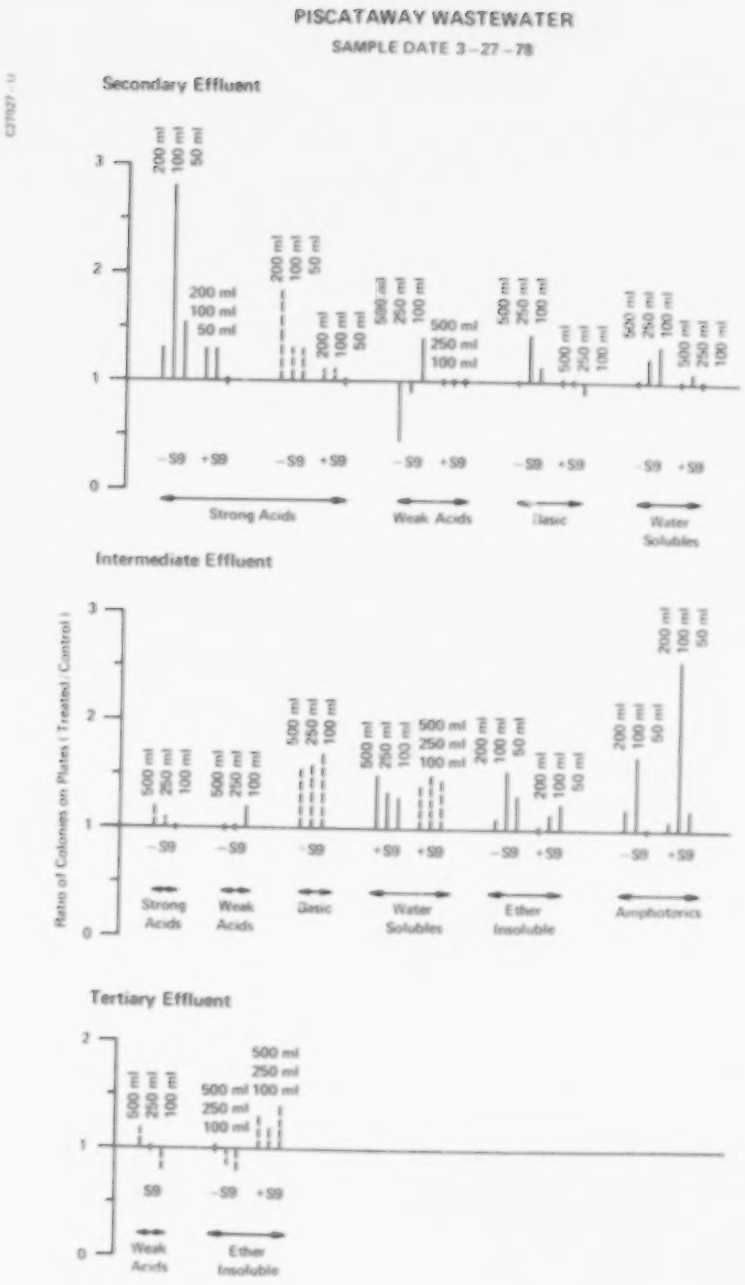
The final AWT effluent, while it was free of any oxygenated mutagens, contained a high level of activity in the aromatic fraction (Figure 20). Since no activity in this fraction was detected in the intermediate effluents, these mutagens appeared to have been introduced during treatment. The aliphatic and the oxygenated neutrals of the AWT effluent at the concentration used were toxic to the bacteria, and, therefore, it cannot be said with certainty if mutagenic compounds were present in these fractions.

The mutagenic responses elicited by the fractionated organics from the second sample of Piscataway wastewater (sample date 5-10-78) are shown in Figures 21 through 24. Because of technical problems, no sample was obtained for solvent extraction. The separated classes of chemicals obtained by XAD resin from the AWT influent showed no mutagenic or toxic response, while that obtained with polyurethane foam showed weak responses in the weak acid,



Salmonella Strain TA98
Salmonella Strain TA100

Figure 16. Dose-related response of the selected separate fractions of the XAD concentrates of Piscataway wastewaters (sample 1)



——— Salmonella Strain TA98
----- Salmonella Strain TA100

Figure 17. Dose-related response of the selected separated fractions of the solvent concentrates of Piscataway wastewaters (sample 1)

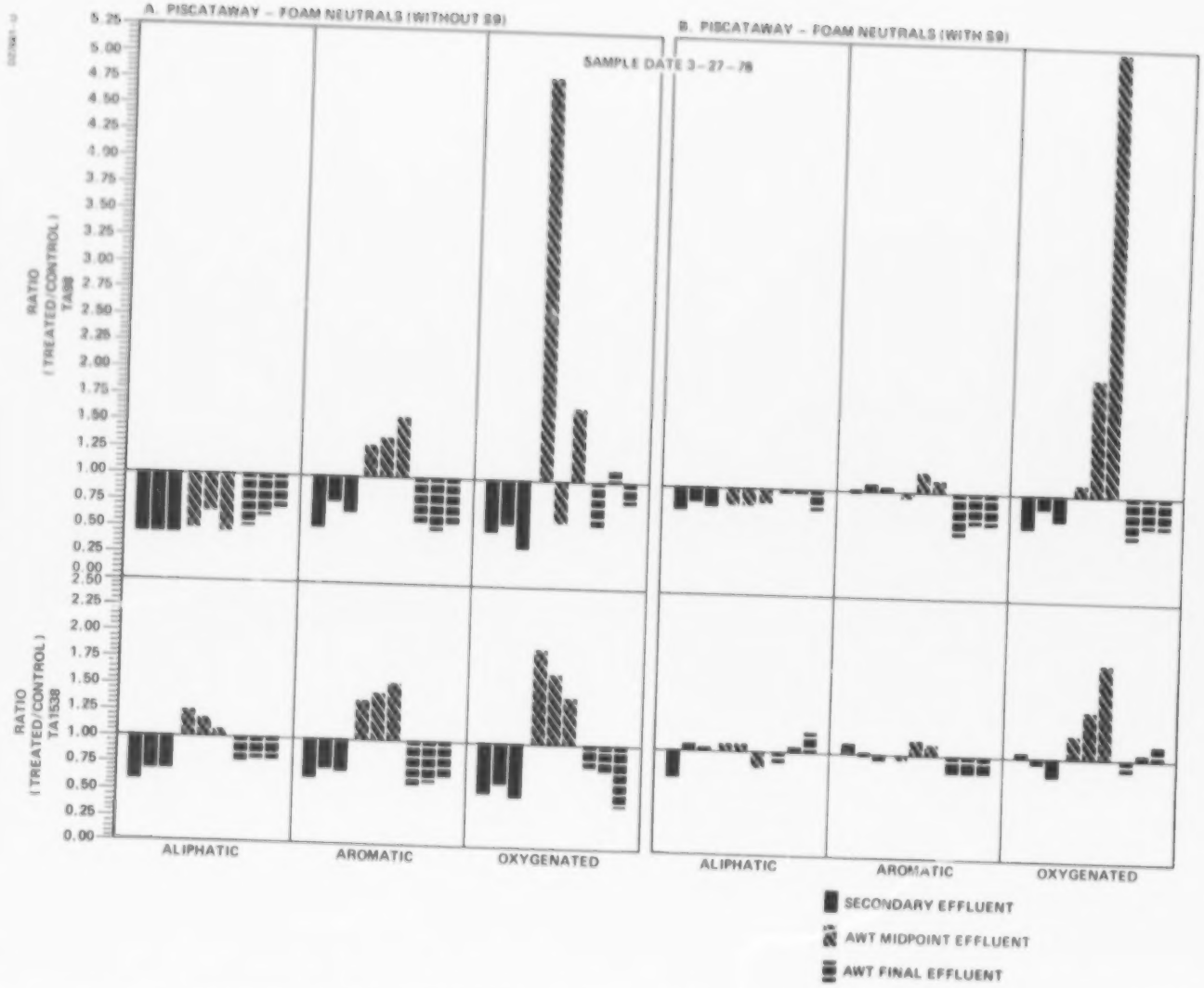


Figure 18. Mutagenic activity of the components of neutral fraction of Piscataway wastewater collected with polyurethane foam as sorbent (sample 1)

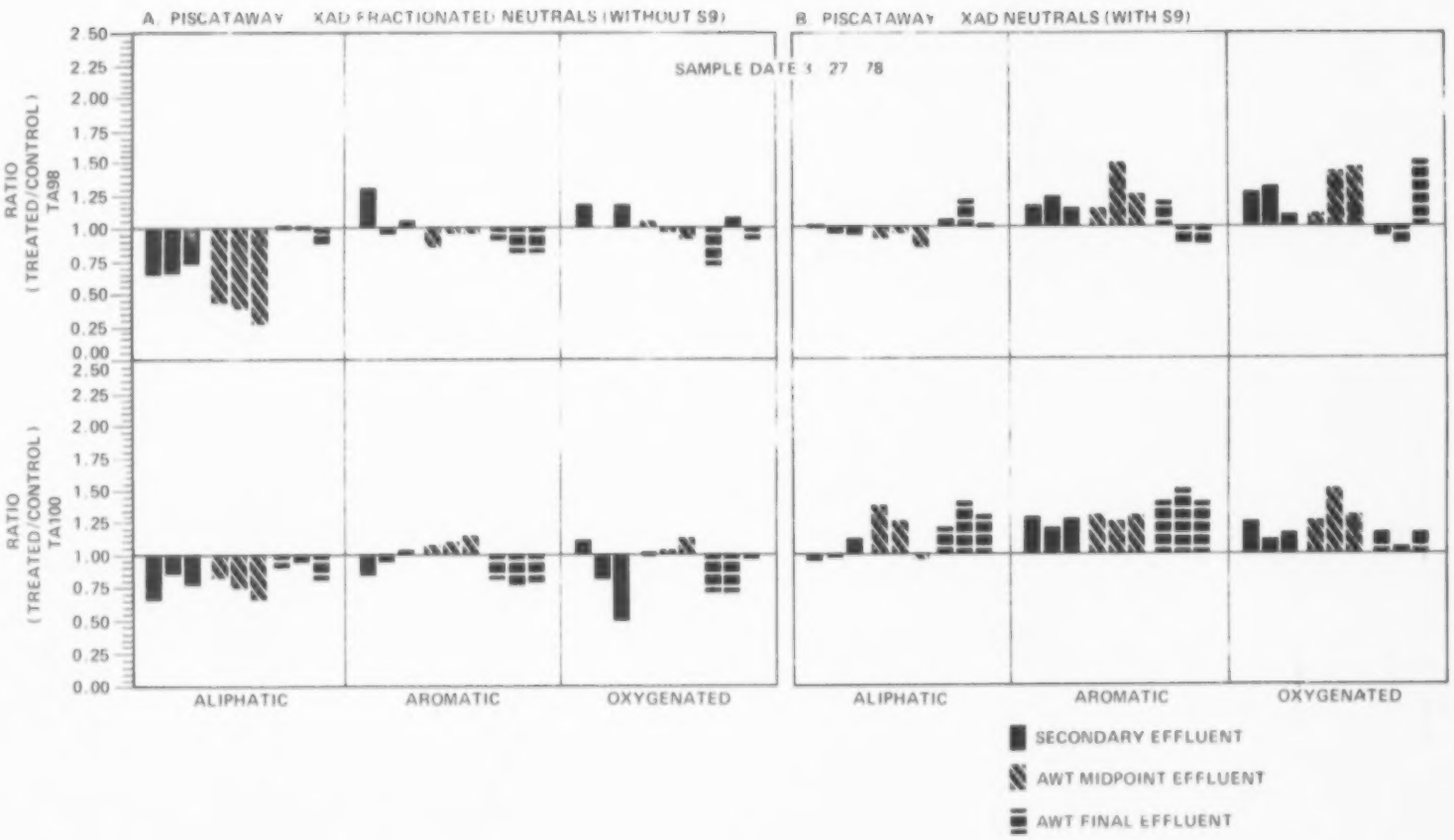


Figure 19. Mutagenic activity of the components of neutral fraction of Piscataway wastewaters collected with XAD as sorbent (sample 1)

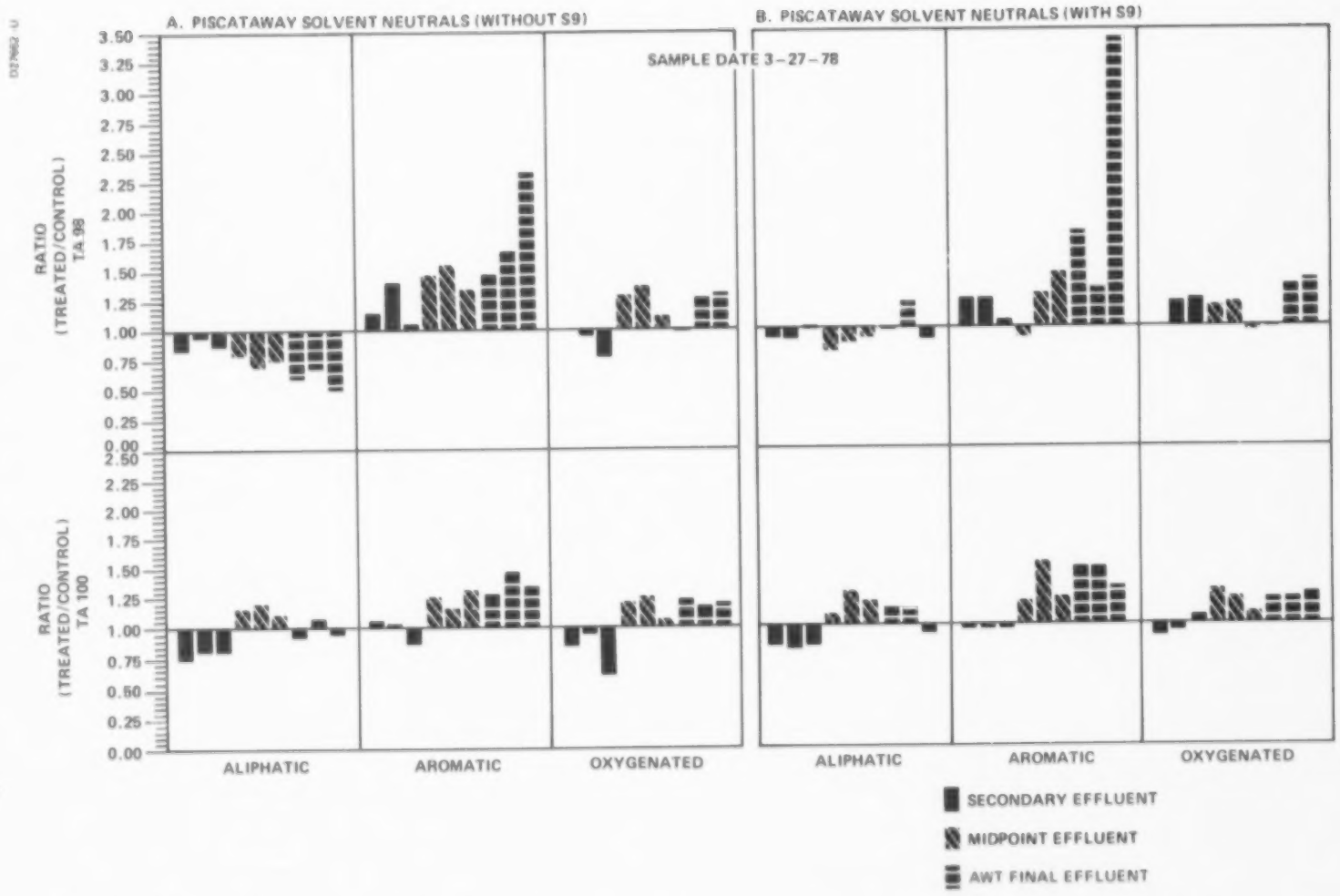


Figure 20. Mutagenic activity of the components of neutral fractionof Piscataway wastewaters recovered by extraction with methylene chloride-hexane (sample 1).

amphoteric, and oxygenated neutral fractions. The mutagenic response was observed with frameshift mutants only and was less than that observed with the previous sample from Piscataway (sampling date 3-27-78), with the highest ratios (experimental/spontaneous) being only 2.1. On the other hand, almost all the separated fractions from the midpoint sample showed activity, many giving ratios well above 2.0. It should be mentioned that a similar increase in the number of mutagenic fractions after liming and recarbonation was also noted with the first sample. The activity was more pronounced in the XAD fractions. Breakpoint chlorination/carbon filtration steps caused a decrease in activity in many fractions, but activity was still detectable in basic, water soluble, strong acid, and aliphatic neutral fractions. It appeared, in fact, that some of the activity detected in the final effluent was contributed by the treatment steps themselves.

The differences and similarities between the two samples from the Piscataway AWT plant are summarized below:

3-27-78 Sample	5-10-78 Sample
1. Mutagenic response was noted in both base pair substitution and frameshift mutants.	1. Mutagenic response was noted only in frameshift mutagens.
2. Mutagenic activity was more pronounced in polyurethane foam fractions.	2. Activity was more pronounced in XAD fractions.
3. Major mutagenic fractions (ratios > 2.0) were: - Strong acids, basic, ether insoluble, week acids and oxygenated neutrals for AWT influent - Strong acids, ether insoluables, basic, amphoterics and oxygenated neutrals for AWT midpoint - Weak acids, aromatic neutrals for AWT final effluent	3. Major mutagenic fractions (ratio > 2) were: - Weak acids, amphoteric and oxygenate neutrals for AWT influent - Strong acids, basic, weak acids, water soluble, and amphoteric for AWT influent - Basic, aliphatic neutrals for AWT final effluent
4. Overall the mutagenic response was of a greater order of magnitude.	4. Overall the mutagenic response was of a lower order of magnitude.
5. Liming and recarbonation caused appearance of weak to moderate activity in ether insoluble (chemical nature different than that noted for influent), water soluble, and amphoteric fractions.	5. Liming and recarbonation caused appearance of activity in almost all of the chemical classes.

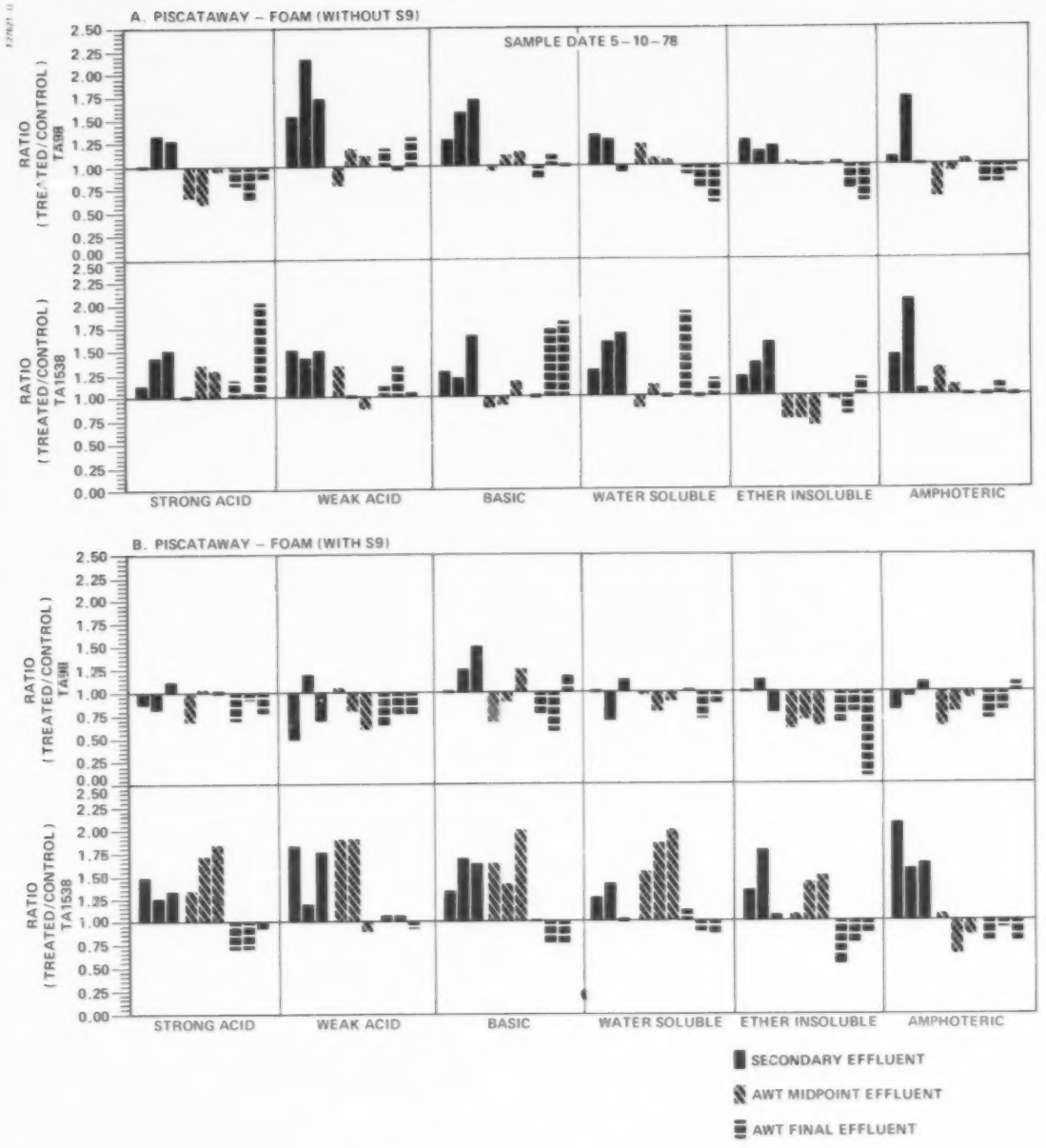


Figure 21. Mutagenic activity of the major classes of compounds recovered from Piscataway wastewaters with polyurethane foam plugs as sorbent (sample 2). The three vertical bars in each set represent three different concentrations of the fraction tested. Concentrations from left to right are: separated fractions representing 100, 250, and 500 ml wastewater.

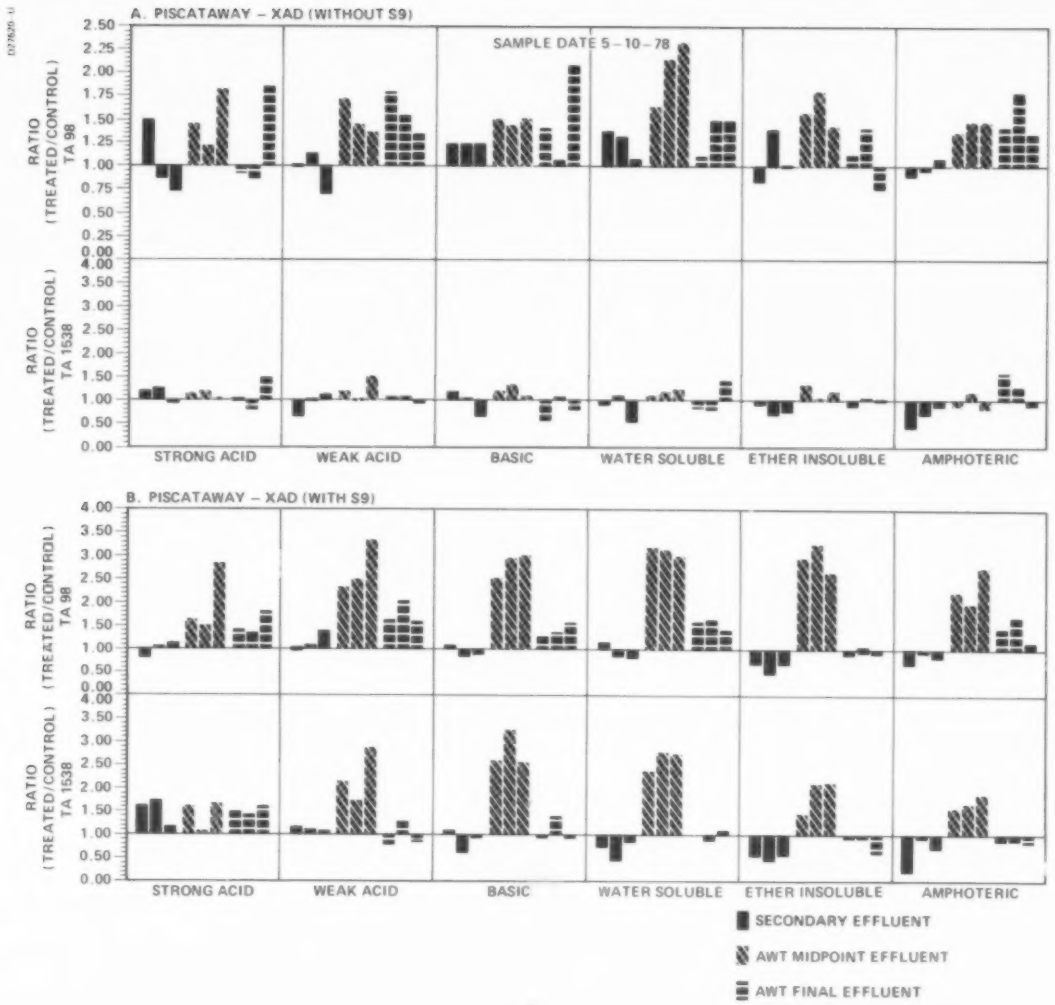


Figure 22. Mutagenic activity of the major classes of compounds recovered from Piscataway wastewaters with XAD resinas sorbent (sample 2). The three vertical bars ineach set represent three different concentrations of the fraction tested. Concentratons from left to right are: separated fractions representing 100, 250, and 500 ml wastewater.

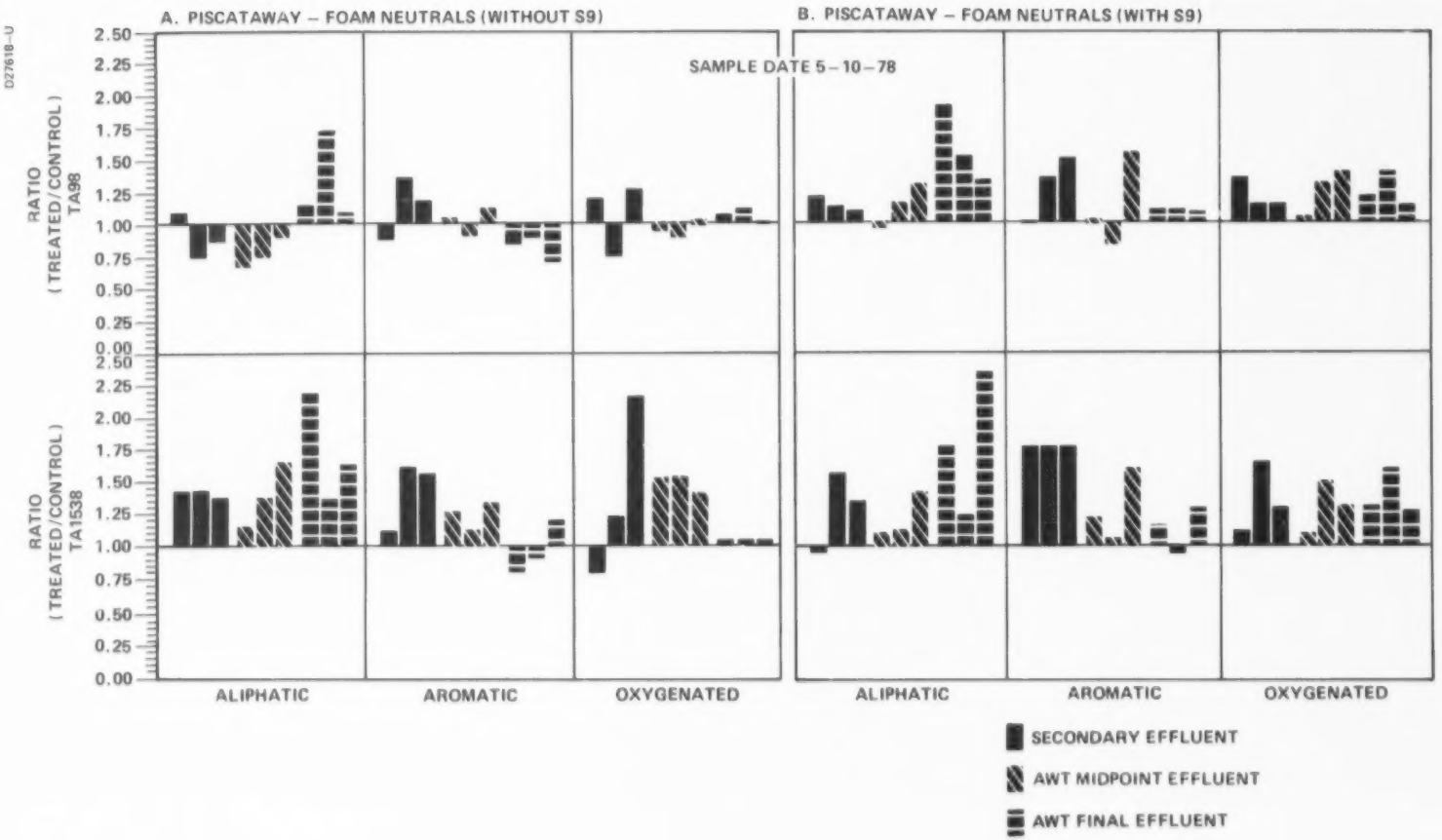


Figure 23. Mutagenic activity of the components of the neutral fraction of Piscataway wastewater collected with polyurethane foam sorbent (sample 2). The three vertical bars in each set represent three different concentrations of the fraction tested. concentrations from left to right are: aliphatic representing 50, 100, and 200 ml wastewater; aromatic representing 50, 100, and 200 ml wastewater; oxygenated representing 10, 25, and 50 ml wastewater.

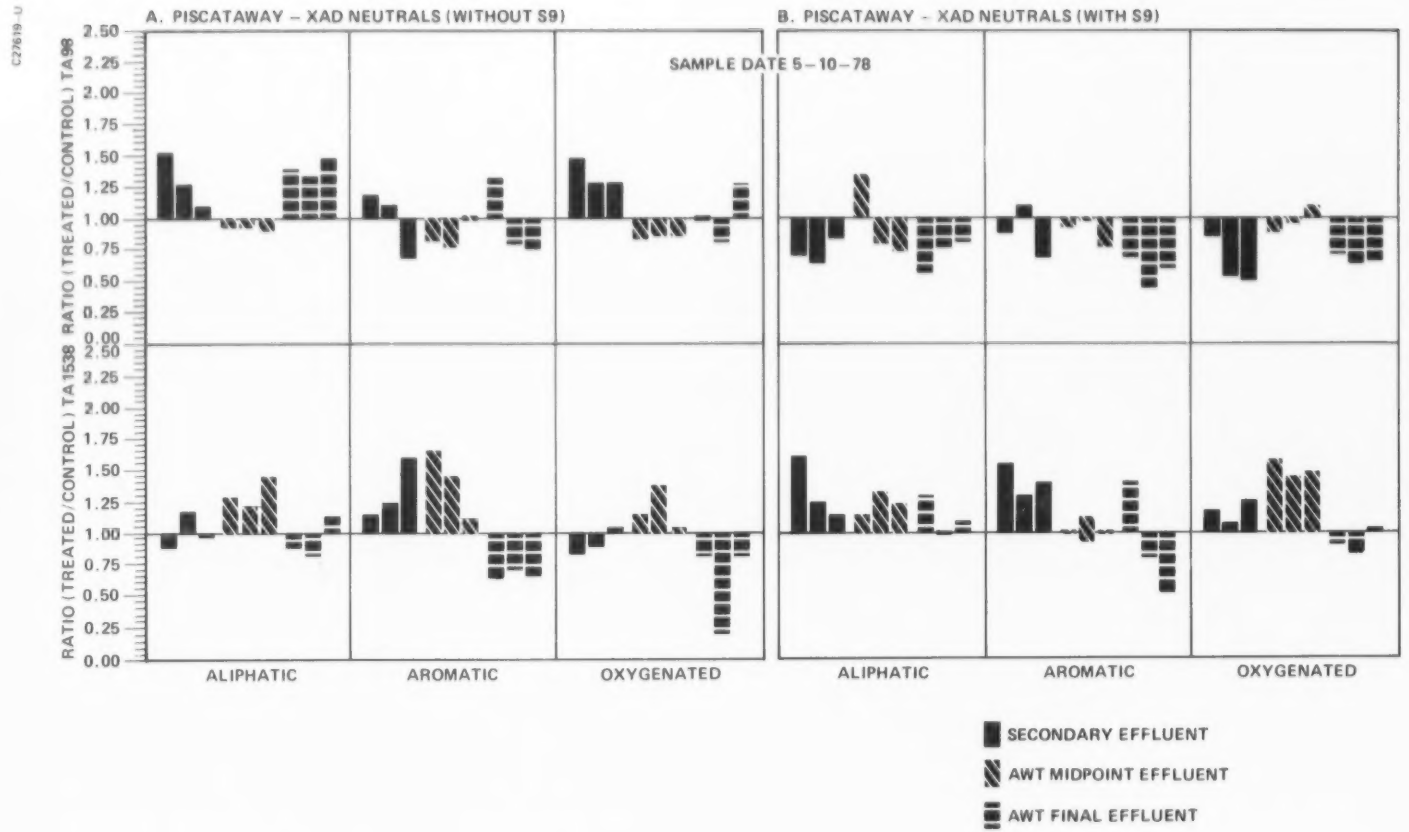


Figure 24. Mutagenic activity of thecomponents of the neutral fraction of Piscataway wastewater collected with XAD as sorbent (sample 2). The three vertical bars in each set represent three different concentrations of the fraction tested. concentrations from left to right are: aliphatic representing 50, 100, and 200 ml wastewater; aromatic representing 50, 100, and 200 ml wastewater; oxygenated representing 10, 25, and 50 ml wastewater.

The qualitative and quantitative differences in the mutagenic response of the two samples from Piscataway probably represent the normal variation in the quality of the influent. It should be noted that the chemical classes in which activity appeared after liming and/or recarbonation also differed considerably for the two Piscataway samples. If the mutagens were introduced as contaminants in chemical additives, such a wide variation from one batch of chemical additives to the next would be highly unlikely. On the other hand, if mutagens were chemically synthesized during liming and/or recarbonation, the qualitative and quantitative variations in the mutagen precursors in the influent wastewater from one time to the next, could explain the observed variation in the mutagenic response of the two samples.

Yeast mutagenesis assay--Forward mutagenesis assays using yeast strain S288C were performed with the separated classes of organics generated from the 3-27-78 sample of Piscataway. Because of the lower sensitivity of the assay, separated fractions were tested at concentrations representing 200 ml wastewater. Secondary effluent elicited a weak response with some fractions showing experimental/spontaneous ratios between 2 and 3 (Figure 25). Similar to the findings with the Ames' assay, the midpoint sample showed a greater number of fractions with ratios above 2.0. Significant activity toward the yeast strain was not noted with the AWT final effluent.

Even though the findings with the yeast mutagenesis assay appear, in general, to corroborate the results with the Ames' assay, it should be stated that there are inherent ambiguities in the forward mutagenesis assay which result from replicate plating. This renders the assay less attractive for detecting low levels of mutagens.

Conclusion

The physicochemical AWT process employed at Piscataway was effectively able to treat many, but not all, classes of mutagenic compounds. Also, some treatment steps, particularly liming and recarbonation, showed a potential for introducing mutagenic substances. The final AWT effluent was found to contain mutagens belonging to the weak acids, basic, aromatic and aliphatic neutral classes, and many highly polar mutagens which could not be recovered by any of the concentration methods employed in the study. The presence of the polar mutagens was demonstrated in the studies with unconcentrated wastewaters.

EVALUATION OF COMBINED BIOLOGICAL AND PHYSICAL-CHEMICAL TREATMENT AT BAY PARK, L.I.

The sampling locations chosen for this facility represented influent and effluent for the AWT process. Secondary effluent from the conventional treatment process served as influent, and the treated wastewater after the chlorination step as effluent. The studies with unconcentrated wastewaters were conducted on two samples collected on individual dates two months apart. However, the studies involving recovery of organics from wastewater and bioassay of the crude concentrates and separated fractions could only be performed on one sample because of the early closing of the AWT facility.

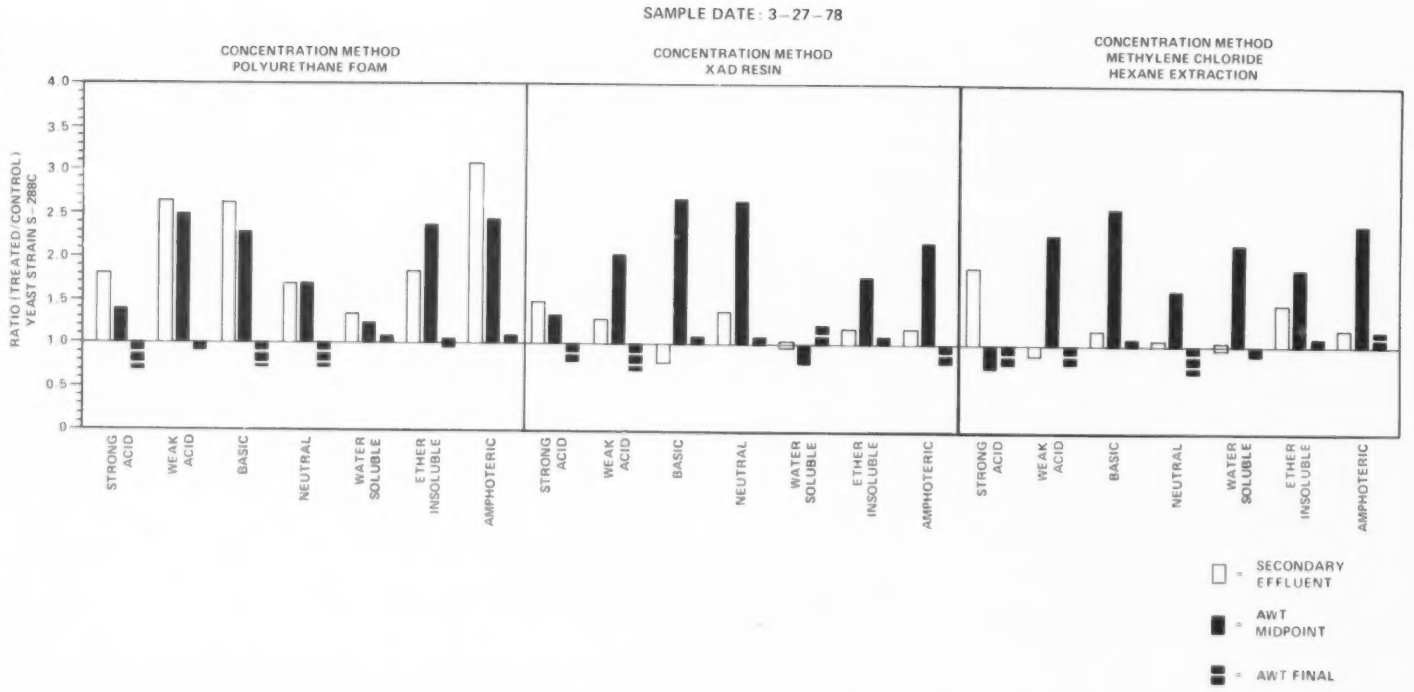


Figure 25. Genetic activity of the separated fraction of Piscataway crude concentrations in S. cerevisiae strain S288C.

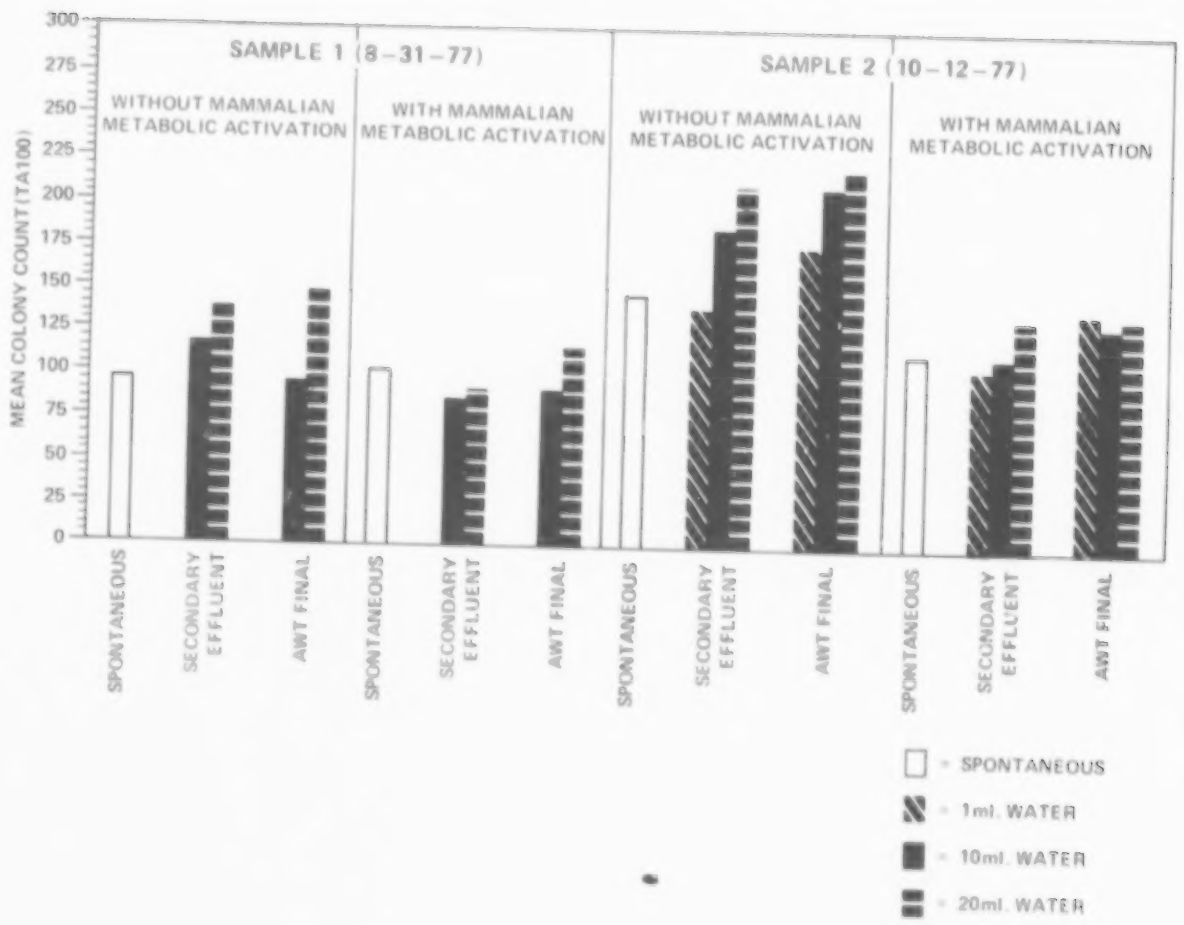


Figure 26. Mutagenic response of the unconcentrated wastewaters from Bay Park AWT Plant with Salmonella strain TA100

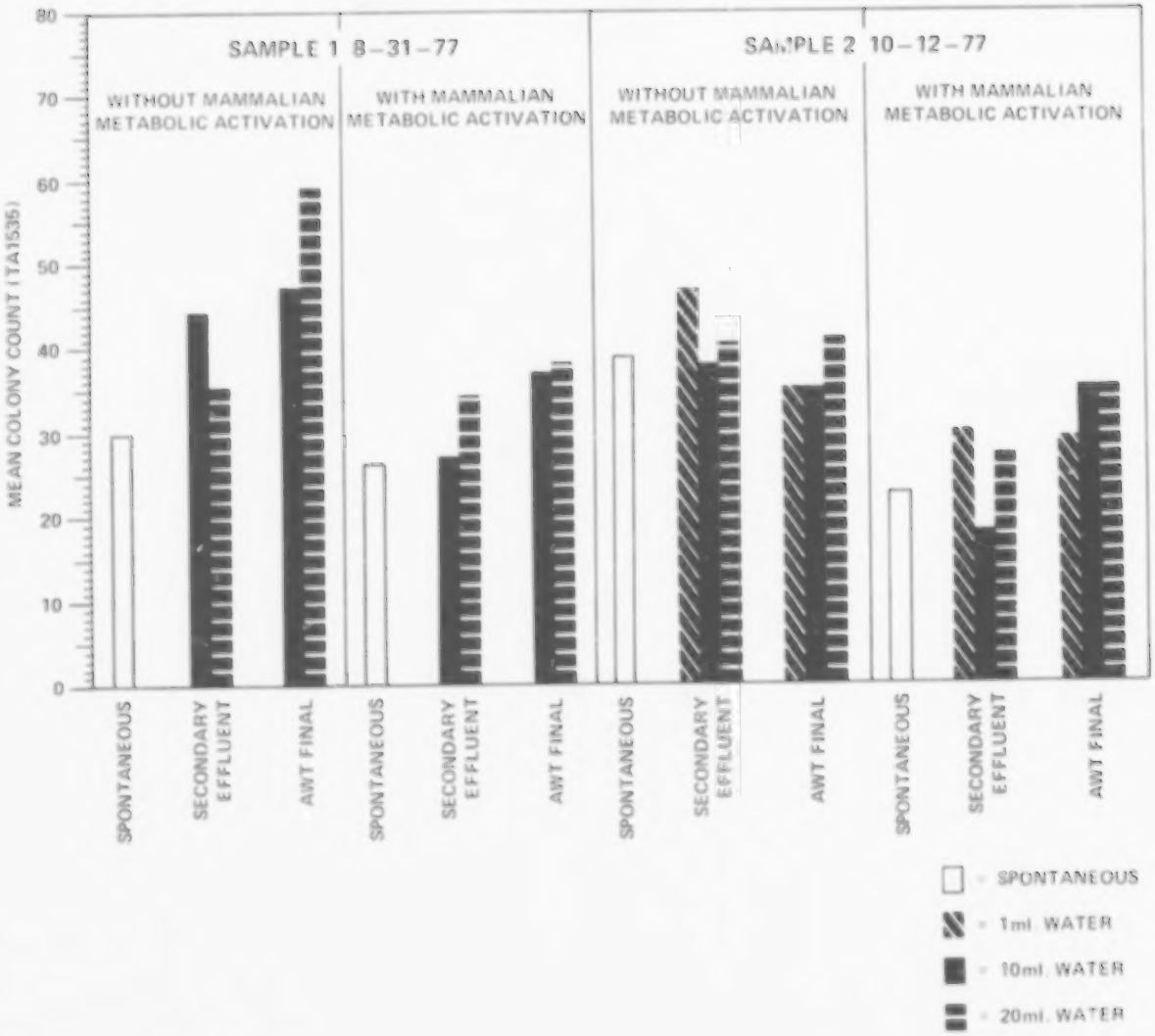


Figure 27. Mutagenic response of the unconcentrated wastewaters from Bay Park AWT with Salmonella strain TA1535

observed clarity of the secondary effluent were apparent during the time necessary to concentrate organics from 60 to 90 ℓ of wastewater. The two TOC values for the secondary effluent represent the samples taken at two different times during the concentration run: first when the effluent was very murky, and again when it was relatively clear. The murkiness of the secondary effluent and its unusually high TOC value (156 ppm) were the result of transient instability at the Bay Park facility. The tertiary effluent showed no substantial variation in the TOC value.

XAD resin and polyurethane foam plugs recovered a very small amount of the carbon present in wastewater. The recovery efficiencies were much lower than those reported earlier for Piscataway wastewaters. The low recovery of organic carbon by the two collection procedures was probably due to the presence of fine organic particulate matter in the wastewaters which was not retained by the concentration procedures. Unlike the heavy floc present in Piscataway wastewaters, the fine particles at Bay Park passed through the glass wool filter and hence contributed to the TOC of the wastewaters.

TABLE 11. RECOVERY OF ORGANIC CARBON FROM BAY PARK WASTEWATERS BY DIFFERENT COLLECTION METHODS

Sample	Total Organic Carbon (ppm)	Percent Carbon Recovered by Foam	XAD	Extraction by Ethylene Chloride-Hexane
Secondary Effluent	156-71*	0.9-2*	1.2-2.7*	ND
Tertiary Effluent	17.6	0.3	0.8	ND

*TOC determination and calculated percents obtained from two grab samples at different time during concentration.

ND = Not determined because of insufficient sample.

Mutagenic response with crude organic concentrates--

Crude organic mixtures recovered from Bay Park wastewater showed mutagenic activity with Salmonella strains TA100 and TA1538 (Figures 28 and 29). The activity was more pronounced with strain TA1538 producing a treated versus spontaneous revertant ratio of greater than 2.0. However, changes in concentration of the organic mixture in the assay failed to produce a dose-related response. The secondary effluent was toxic to many strains with the solvent concentrates causing the greatest toxicity, followed by polyurethane foam and XAD. Although toxicity was relieved by the presence of liver enzymes in the assay, the enzyme system was not capable of completely detoxifying the sample. The final effluent showed considerably less toxicity than the secondary effluent.

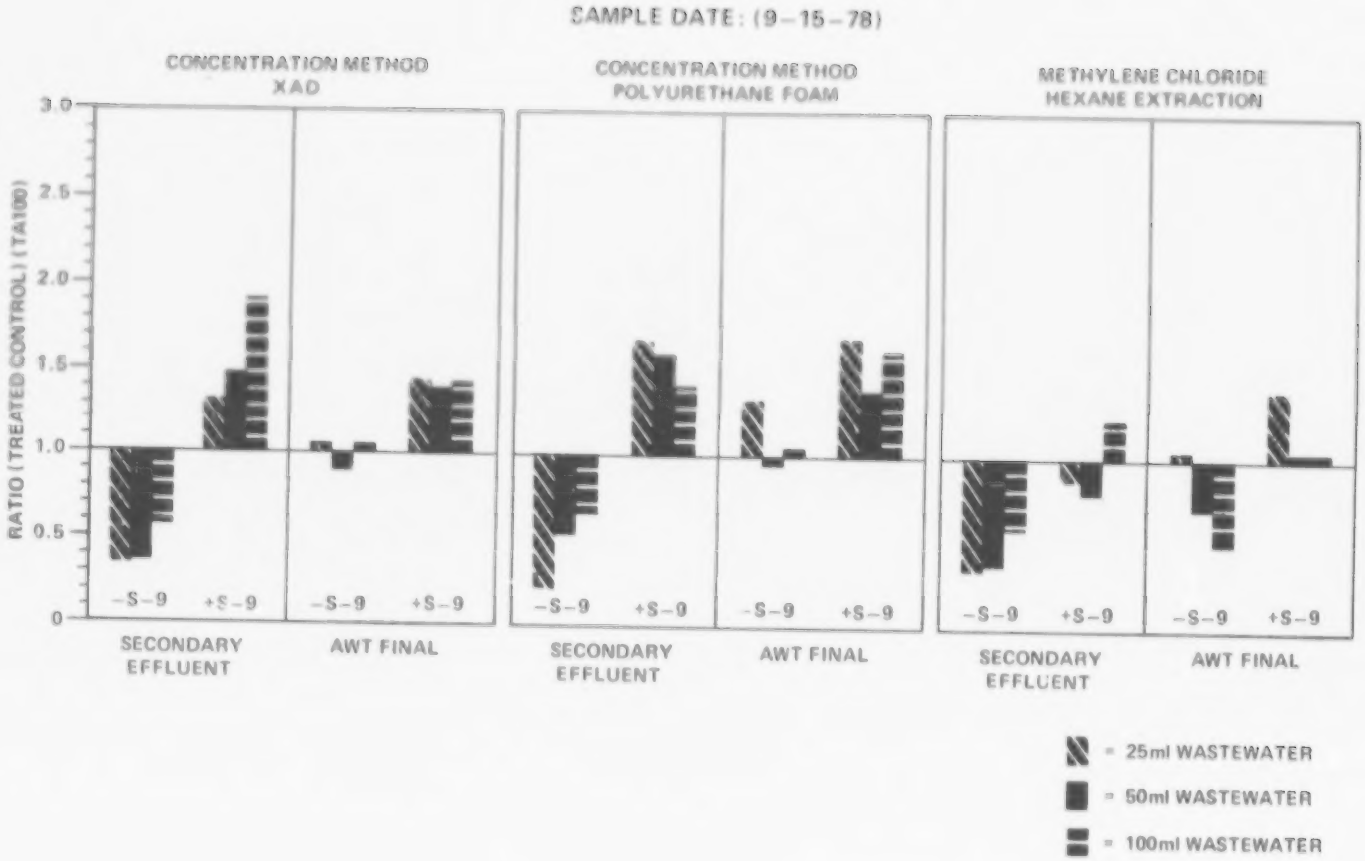


Figure 28. Mutagenic response of the crude organic mixture recovered from Bay Park wastewater with Salmonella strain TA100

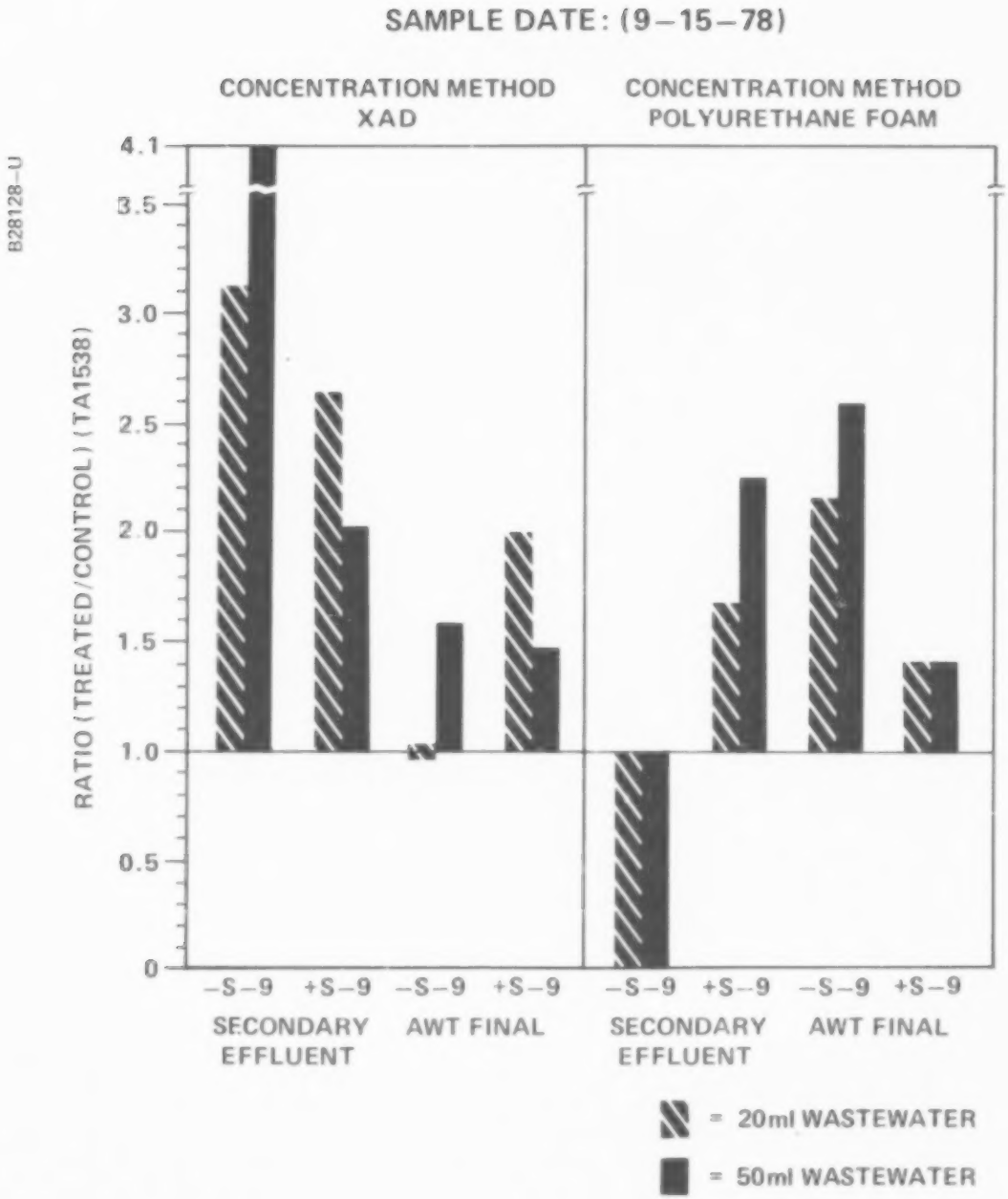


Figure 29. Mutagenic response of the crude organic mixture recovered from Bay Park wastewater with Salmonella strain TA1538

The yeast forward mutagenesis assay on the Bay Park concentrates revealed no genetic activity in the sample at concentrations of up to 400 ml wastewater/assay. The AWT final effluent concentrates proved to be severely toxic to yeast at these concentrations as shown by reduction in the spontaneous mutation rates.

The low mutagenic response of the Bay Park concentrates and the fact that the percent recovery of carbon by the concentration methods was usually low (Table 11), raised the possibility that the concentration methods may be ineffective in concentrating mutagens from these wastewaters. Studies were carried out to ascertain if the concentration methods were leaving mutagens in the wastewaters in large amounts. The effluent samples after each concentration procedure were dried by rotary evaporation, and organics were recovered from the crude extract by extraction with methanol. The organic mixture was solvent exchanged with DMSO and assayed using the Ames' assay. After filtration through polyurethane foam or XAD resin, significant residual mutagenic activity was not detectable in the secondary effluent. Tertiary effluent treated in a similar manner showed marginal mutagenicity (Ratio 1.1-1.7) with Salmonella strain TA100. However, a significant amount of residual activity was detectable in the secondary and tertiary effluents after they had been subjected to the solvent extractions. Increase in the reversion rates was of the order of 2.5 to 3.3, and was observed with Salmonella strains TA100 and TA98. Metabolic activation was necessary for mutagenic activity. The results show that polyurethane foam plugs and XAD resin were able to effectively concentrate mutagens from wastewaters. Solvent extraction, on the other hand, was unable to recover all the activity. The superior performance of polyurethane foam plugs over solvent extraction was also shown in the earlier studies reported with Piscataway wastewaters.

Chemical Separation of Crude Organic Concentrates and Bioassay of Separated Fractions

Chemical Separation of Crude Concentrates--

The distribution of organic carbon recovered by polyurethane foam and XAD resin from Bay Park influent and effluent, into different chemical classes is shown in Table 12. Similar to the results with Piscataway wastewaters, the sum of carbon in separated fractions was greater than that detected in the crude concentrates. It should be restated that the data can be used only to derive trends. Because of insufficient volume, the solvent extract was not fractionated. Limited availability of material also prevented TOC measurement of the neutrals recovered by foam plugs.

XAD resin collected basic, water soluble, and ether insoluble compounds with higher efficiency than polyurethane foam. This is in agreement with the reported ability of XAD resin to collect organic acids, bases, and other relatively polar compounds from water (Webb, 1975). Comparison of the recovery efficiencies of foam plugs and XAD for neutral compounds cannot be made, because of nonavailability of the data for foam plugs. Based on the data from Piscataway wastewaters, it can be speculated that foam plugs collected neutral compounds more efficiently than XAD resin. Comparison of TOC in the separated fractions of influent and effluent

TABLE 12. MASS DISTRIBUTION OF THE CRUDE ORGANIC MIXTURE RECOVERED BY POLYURETHANE FOAM AND XAD RESIN FROM BAY PARK WASTEWATERS

Sample	Concentration Method	TOC in Separated Fractions (ppm)								
		Strong Acids	Weak Acids	Basic	Water Soluble	Ether Insoluble	Amphoteric	Aliphatic Neutrals	Aromatic Neutrals	Oxygenated Neutrals
Secondary Effluent	Polyurethane Foam	0	0.0915	0.079	0.0028	0.092	0	+	+	+
	XAD Resin	0.135	0.167	0.897	0.41	0.35	0.0047	0.22	0.23	0.7
AWT Final Effluent	Polyurethane Foam	0	0.084	0.081	0.033	0.03	0.016	+	+	+
	XAD Resin	0.032	0.112	0.02	0.049	0.06	0.024	0	0	0.05

+Not tested due to insufficient material.

samples revealed drastic reduction in carbon in all the fractions after treatment. Since there was no midpoint sample at this AWT facility, it was not known if any of the treatment steps introduced carbon in the effluent, and if the carbon present in the final effluent represented compounds which may have been added during treatment or compounds which were originally present in AWT influent.

Bioassay of Separated Fractions--

The mutagenic activity of the separated fractions was noted largely in Salmonella strains TA100 and TA98. The results from various separated fractions are shown in Figures 30 and 31. Because of their highly toxic nature, the neutral fractions were subfractionated prior to bioassay.

The mutagenic compounds found in secondary effluent were in the basic, water soluble, and amphoteric fractions. The water soluble mutagens were recovered by XAD resin, and the amphoteric, by solvent extraction. Mutagens found in the basic fraction could be recovered by both concentration methods. Polyurethane foam plugs, on the other hand, failed to recover any class of mutagens from the secondary effluents. Mutagenic responses of the basic, water soluble, and amphoteric compounds were partially reduced after advanced wastewater treatment. At the same time, however, mutagenicity appeared in new classes of chemicals. For example, weak acid and ether insoluble fractions of the secondary effluent were nonmutagenic but acquired mutagenicity after treatment. In additon, a low level of mutagenicity also became visible in the water soluble fraction of the polyurethane foam, and solvent concentrates. These water soluble mutagens appeared to be different from those detected in the secondary effluent, since they could be recovered by polyurethane foam sorption and solvent filtration. The need for using a variety of concentration methods when examining wastewaters for the presence of mutagens was illustrated by the appearance of positive responses in a given fraction for one concentration method but not for the other method. The addition of mutagenic compounds was consistent with the earlier findings with the Piscataway AWT process. Rappaport et al. (1979) in an investigation of primary, secondary, and post- secondary effluent from six treatment plants have reported a correlation between the inability to demonstrate mutagenic response and the toxicity of the sample. Thus, even slight toxicity may mask a mutagenic response. These findings are directly relevant to our results since the fractions which showed an increase in mutagenicity in the final effluent also showed weak toxicity in the secondary effluent (Figures 30 through 32). Thus, mutagens may have been present in the secondary effluent but could not be seen because of toxicity. The AWT process, by removing toxicants, may have permitted their detection. Therefore, the mutagens detected in the AWT effluent may not necessarily have been introduced during treatment, as it might appear. The actual mechanism for increased mutagenic activity in the AWT effluent must await further investigation.

The mutagenic response, with and without the addition of S-9 Mix, for the fractionated neutral compounds of the XAD resin concentrates are shown in Figure 31. In the absence of metabolic activation, toxic effects were observed even at the lower concentration, particularly with strain TA98.

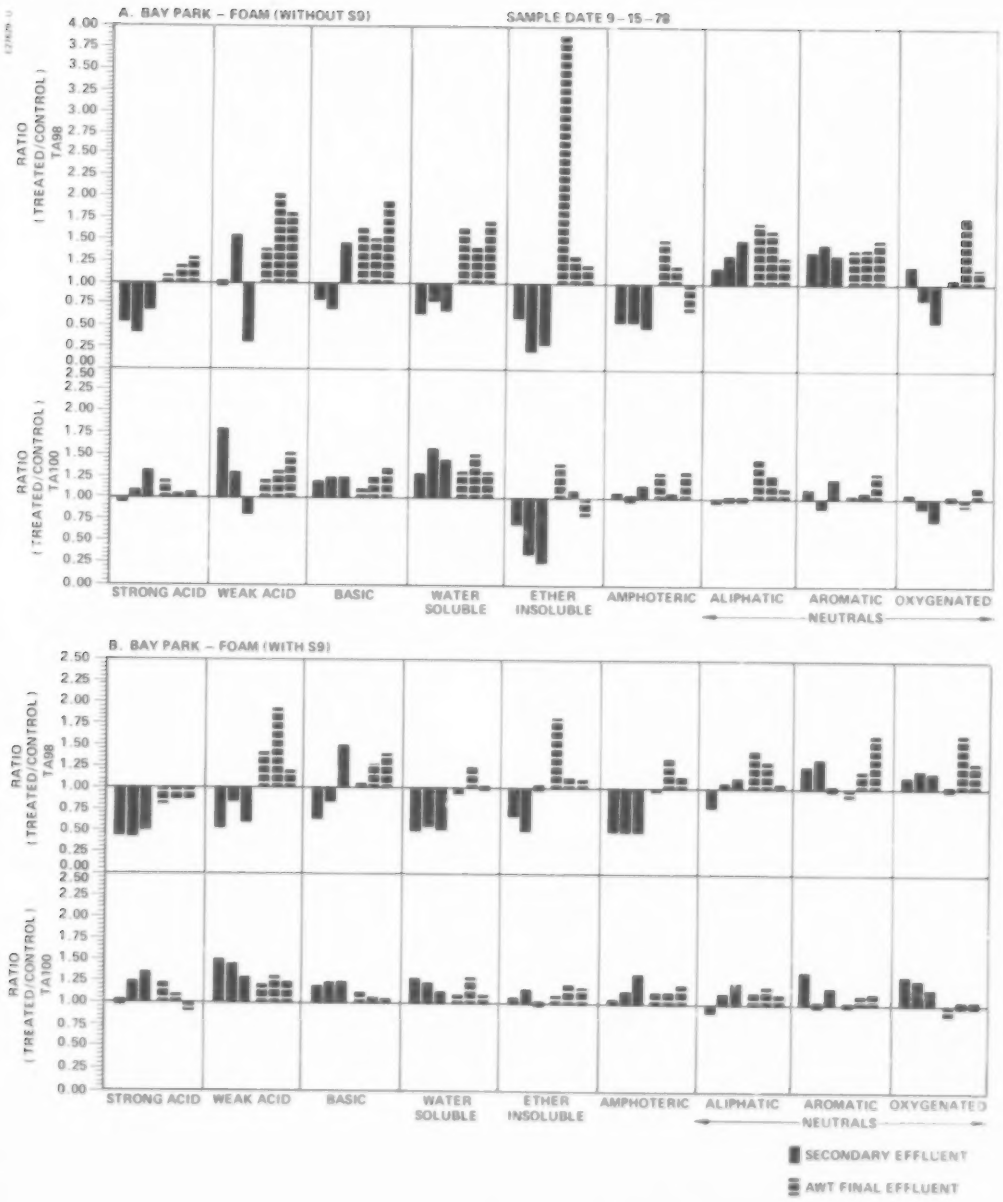


Figure 30. Mutagenic activity of the major classes of compounds recovered from Bay Park wastewater with polyurethane foam plugs as sorbent. The three vertical bars in each set represent three different concentrations of the fraction tested. The concentrations from left to right are: 100, 250, and 500 ml wastewater for all fractions except oxygenated neutrals. For oxygenated neutrals the bars from left to right represent wastewater equivalent to 10, 25, and 50 ml wastewater.

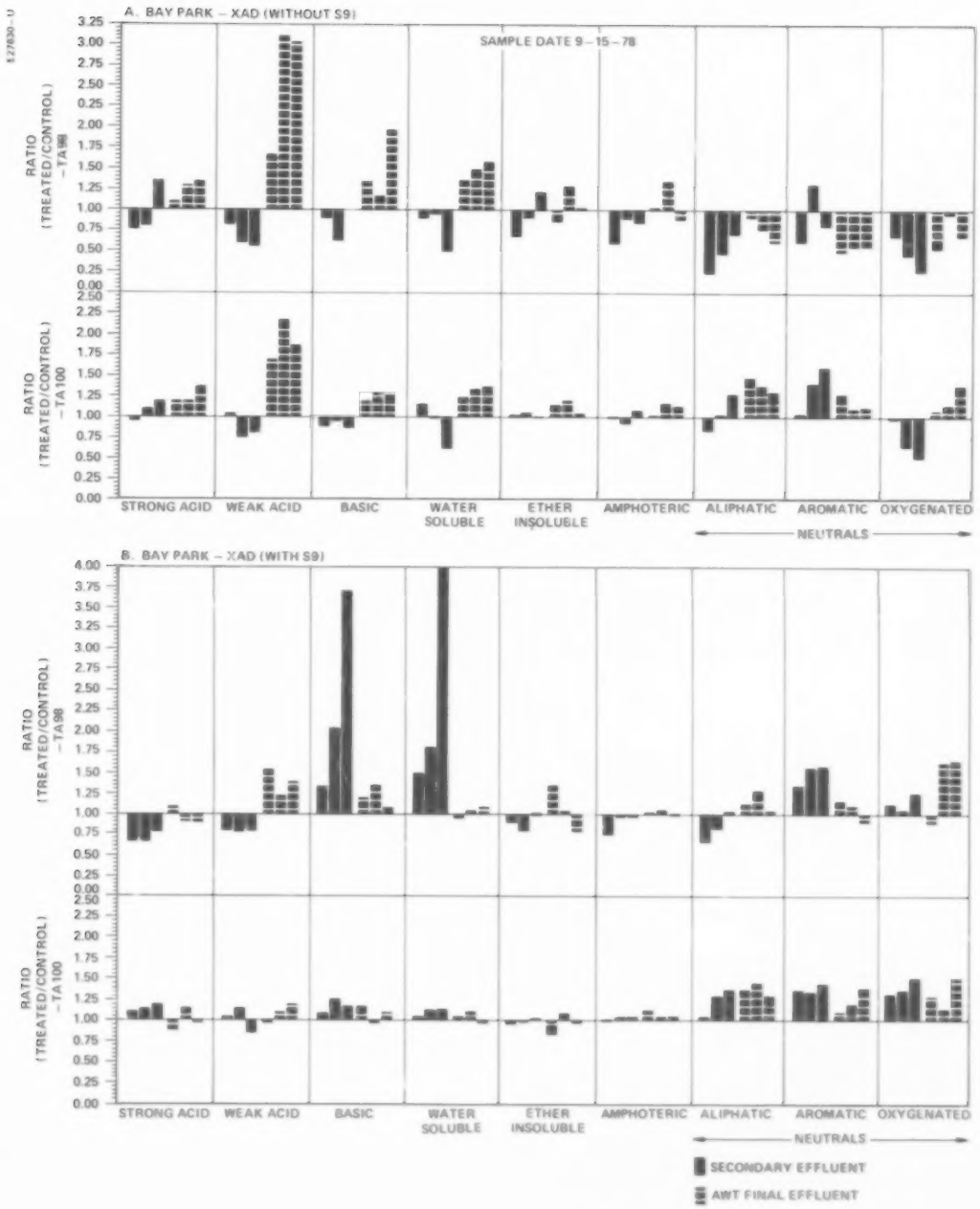


Figure 31. Mutagenic activity of the major classes of compounds recovered by Bay Park wastewater with XAD as sorbent. The three vertical bars in each set represent three different concentrations of the fraction tested. For details of concentrations refer to Figure 30.

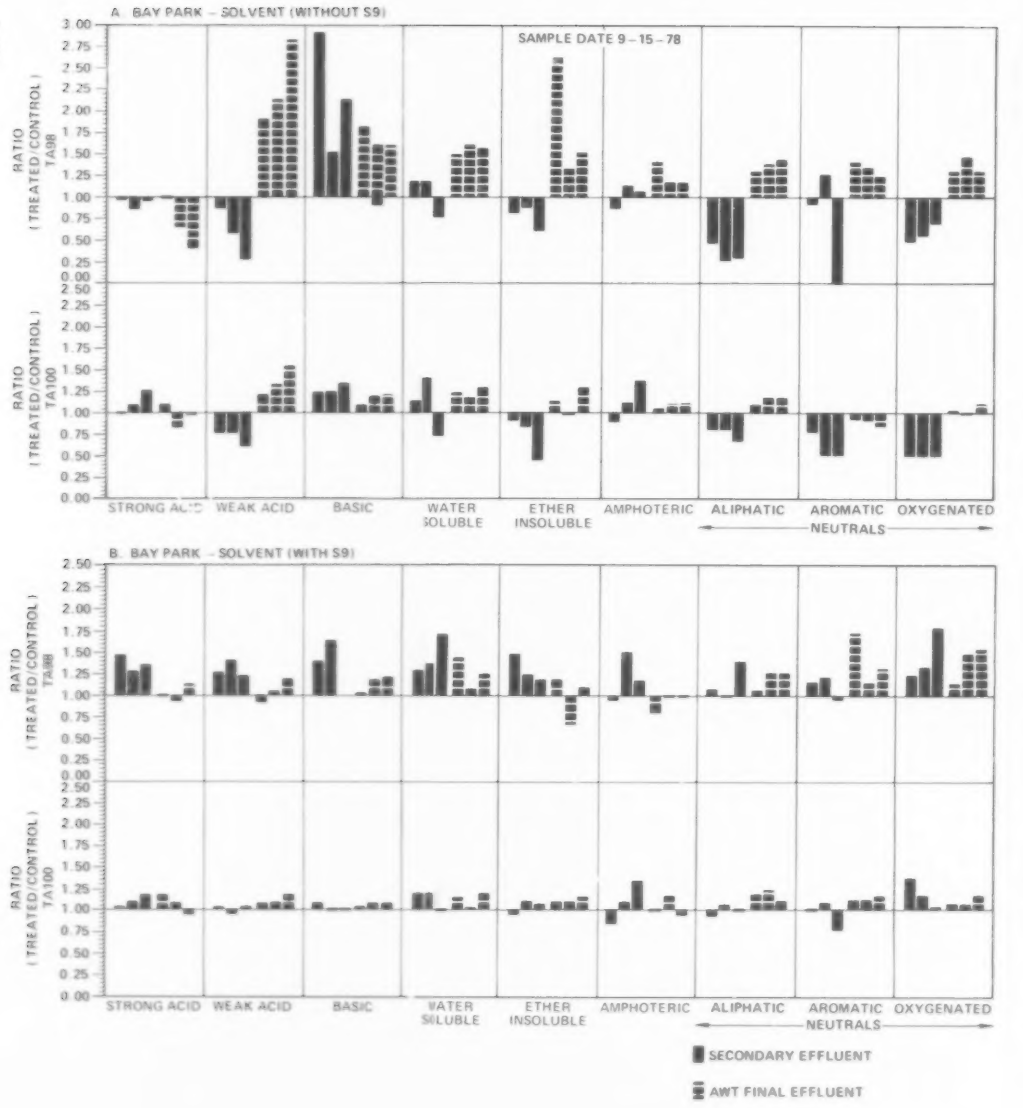


Figure 32. Mutagenic activity of themajor classes of compounds recovered from Bay Park wastewater by methylene chloride-hexane extraction. The three vertical bars in each set represent three different concentrations of the fractions tested. For details of concentrations refer to Figure 30.

The oxygenated fraction was far more toxic than any of the other fractions. Metabolic activation again relieved the toxic effect. The aliphatic, aromatic, and oxygenated neutral compounds showed a weak mutagenic response (ratios <2) with Salmonella strains TA98 and TA100. The activity was detected in the fractions recovered from secondary, as well as AWT final effluent from Bay Park. XAD was the least effective in concentrating mutagenic neutral compounds, but recovered toxicants from the wastewaters with high efficiency. It appears likely that the mutagenic response of the neutral fractions of XAD concentrate may have been masked by the toxicants.

Unlike the results reported above with the Ames' Salmonella assay, none of the chemical classes of Bay Park concentrates exhibited genetic activity in the S. cerevisiae forward mutagenesis assay. The ratio of experimental versus spontaneous revertants for different classes ranged from 0.5 to 1.5. Negative results were obtained with the separated fractions even at concentrations representing 250 mℓ wastewaters. It should be of interest to mention that the separated fractions showed positive response in the Ames' assay at concentrations ranging from 25 to 100 mℓ wastewaters.

Conclusion

The AWT effluent from Bay Park Water Reclamation Plant was shown to contain mutagens belonging to weak acids, ether insoluble, and water soluble classes. It appears that some of the mutagens detected may have been introduced during the renovation process. Alternatively, by removing toxicants, the renovation process may have made it possible to detect mutagens originally present in the secondary effluent. It would be prudent to determine if mutagens still remain at monitoring wells in the area.

EVALUATION OF PHYSICAL-CHEMICAL TREATMENT AT NIAGARA FALLS, NY

The treatment process at Niagara Falls, NY, was of particular interest because approximately 60% to 70% of the influent flow consists of industrial waste. Also, this plant essentially entails AWT technology applied to primary effluent, unlike the more common situation where an AWT process is used for further improving the quality of secondary effluent. The sampling locations for this plant were: influent (primary effluent after flocculation, sedimentation, and acid mixing), midpoint sample (after carbon contact), and final effluent (after chlorination). Due to major mechanical problems, particularly with the carbon filtration process, the AWT facility was in normal operation only for a short period of time, and only one study with unconcentrated wastewaters could be performed.

Wastewaters from all three sampling points showed weak to moderate mutagenicity with base pair substitution and frameshift tester strains of S. typhimurium (Figures 33 and 34). While mammalian metabolic activation was required for the frameshift strains TA98 and TA1538, its presence in the assay caused a decrease in mutagenesis with base pair substitution strains TA100 and TA1535. The requirement for metabolic activation for expression of mutagenic activity suggests the presence of promutagens in wastewater. Alternatively, the activation system, by detoxifying non-mutagenic toxicants and/or masking agents, may have permitted expression of

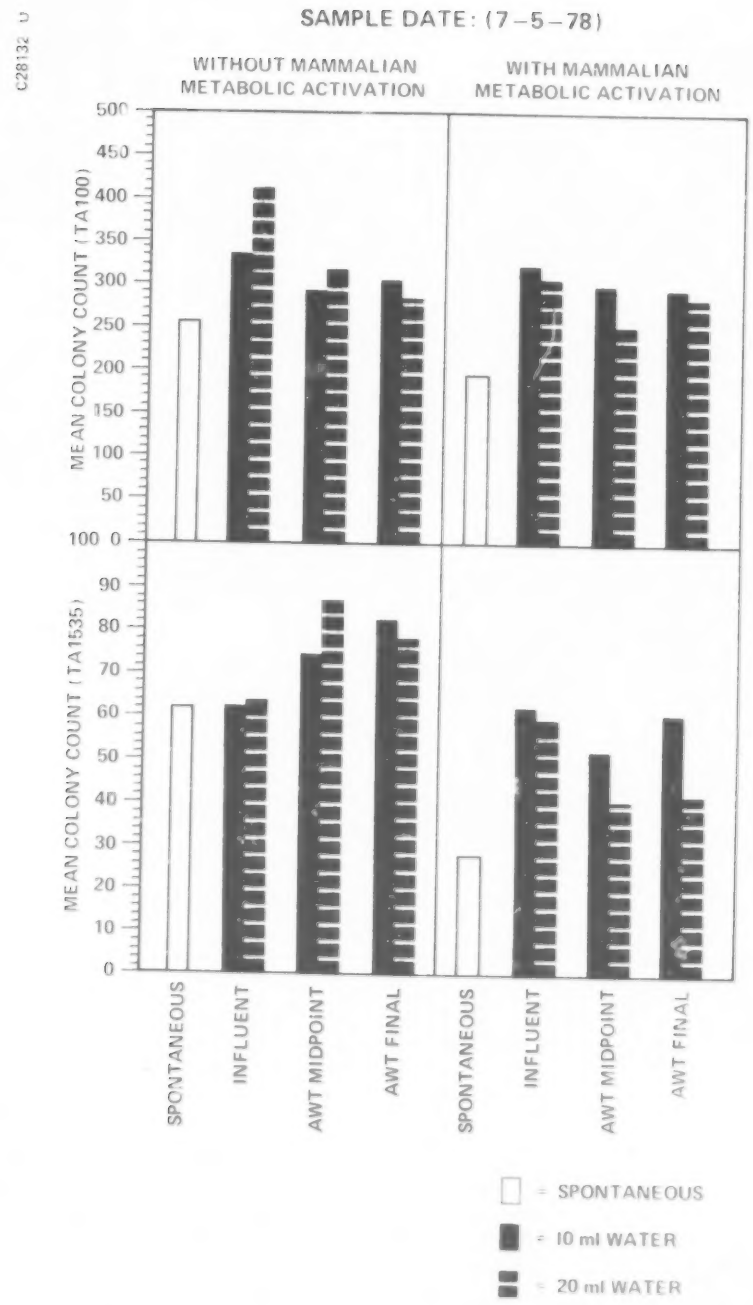


Figure 33. Mutagenic response with Niagara Falls unconcentrated wastewaters with Salmonella strains TA100 and TA1535

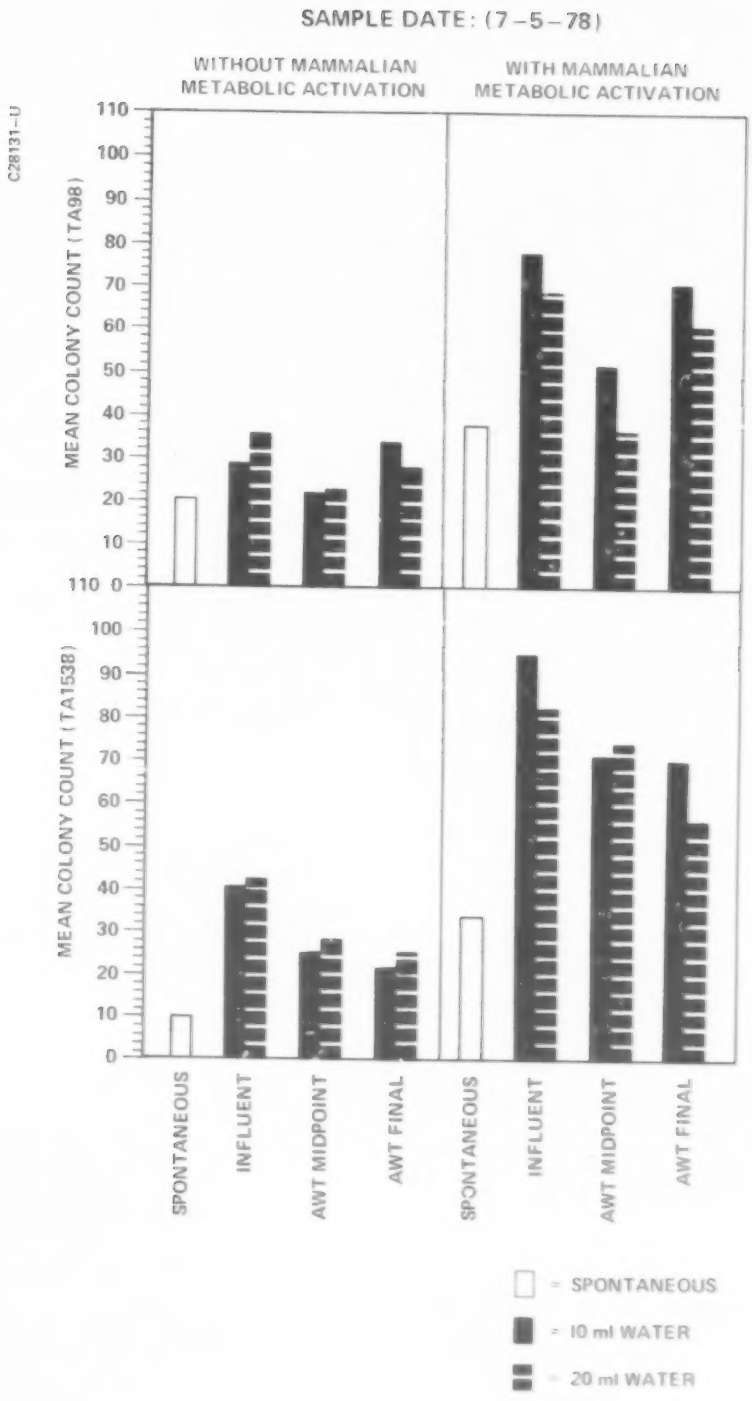


Figure 34. Mutagenic response with Niagara Falls unconcentrated wastewaters with Salmonella strains TA98 and TA1538

mutagens. The presence of toxicants in wastewater at high concentration was also indicated by the fact that an increase in wastewater sample volume in the assay was often accompanied by a decrease in mutagenic response.

The influent wastewaters from this facility were generally more mutagenic than the partially treated and final effluents. This was contrary to the findings with Piscataway wastewaters where the influent was virtually nonmutagenic but acquired mutagenicity during treatment, particularly during the liming and/or recarbonation process. It should be of interest to mention that the Niagara Falls influent samples had undergone partial treatment which included liming and acid addition. Thus, the results with Niagara Falls lend further evidence for the possibility of mutagen/carcinogen addition during the liming and/or pH adjustment step. Another explanation for high mutagenic activity of the influent wastewaters from Niagara Falls may be the presence of large amounts of industrial effluent in these wastewaters. Since there was no sampling point before liming/acid addition, it is not possible to make a judgement on the two possible explanations.

Unconcentrated wastewaters from Niagara Falls failed to induce reverse mutagenesis and mitotic crossover in yeast but produced a weak response in the forward mutagenesis assay. The increase in the number of canavanine-sulfate resistant colonies in the presence of secondary, midpoint, and final effluent (16.5 m wastewater/assay) ranged from 1.5 to 2.0 fold.

EVALUATION OF RAPID SAND FILTRATION PROCESS AT LAKE GEORGE, NY

A detailed investigation of the ability of the rapid sand filtration process at Lake George Village Sewage Treatment Plant (LGVSTP) to remove or introduce mutagenic/carcinogenic substances was undertaken. Two different studies were performed: (a) field study which involved bioassay of the trickling filter effluent (influent for sand beds) and purified effluent collected from different depths of selected sand beds to assess the ability of the sand filtration process to remove or introduce mutagenic carcinogenic substances; and (b) laboratory simulation of the rapid sand filtration process which involved the use of the sand columns available at Rensselaer Polytechnic Institute at Troy, NY, and was performed, because the field study failed to provide the needed information regarding the ability of the rapid sand filtration process to remove mutagenic substances. This information could not be obtained since the influents for sand beds were repeatedly found to be free of mutagens. Also, the sample volumes of purified effluent obtained with the help of lysimeters were relatively small, precluding the collection of a sufficient volume of effluent for concentration and bioassay. These problems were met by dosing laboratory sand columns with wastewaters in which the presence of mutagens had been established (for example, wastewater from Niagara Falls). The size of the effluent sample was predetermined as a function of the amount of wastewater applied to the column, and it was possible to collect samples from different depths of sand.

Field Study: Lake George Rapid Sand Filtration Process

Unconcentrated influent and purified effluent collected from different

depths of the sand beds failed to cause mutagenesis in Salmonella strains tested but showed an inhibitory effect on the spontaneous reversion rate of TA98. Mutagenic response was not elicited after incorporation of β-glucuronidase in the assay suggesting that mutagens were not masked as conjugates. The sample also failed to induce forward or reverse mutagenesis or mitotic gene conversion in S. cerevisiae in the media water assay.

In an attempt to demonstrate whether or not trace amounts of mutagens were present, organics were recovered from the influent and effluent for the sand filtration process and subjected to the bioassay. Only the polyurethane foam concentration method was used because of the limited amount of sample available. This method was chosen, because of the efficiency it showed in collecting mutagens from other wastewaters, and because polyurethane foam generally appeared to collect fewer toxicants. The concentrates were subjected to solubility separation, and the separated fractions were assayed for mutagenicity using the Ames' Salmonella assay at concentrations representing 175 and 350 mℓ wastewaters. Assays were not performed with unfractionated concentrates due to insufficient volume. The lack of sufficient test material also precluded TOC measurement on the separated fractions. The weak acid fraction of the purified effluent showed weak mutagenicity with Salmonella strain TA100 in the presence of mammalian activation system (treated versus control ratio of 2.1 to 2.3). The corresponding fraction from the secondary effluent showed no mutagenic activity suggesting that the weak acid fraction acquired mutagenicity during the sand filtration process. Alternatively, the mutagens may have been present in the influent but could not be seen in the presence of toxicants and/or masking agents. The sand filtration, by removing these agents, may have permitted expression of the mutagenic response. None of the other separated classes of compounds showed significant mutagenic activity, with only a few yielding ratios as high as 1.1 to 1.5.

The absence of significant mutagenic response in the LGVSTP secondary effluents following prolonged contact with the natural delta sand beds could be interpreted to mean that microbial synthesis of mutagens does not occur during wastewater renovation by this process. Alternatively, suitable mutagen precursors and/or cofactors needed for the synthesis of mutagens may be absent from the secondary effluents. It should be emphasized that the LGVSTP wastewater did not contain any industrial wastes; only domestic wastes were present. Strictly domestic wastes generally do not cause mutagenicity. The data does not reveal anything concerning the efficiency of the process in removing mutagenic chemicals because of the absence of such compounds in the wastewaters subjected to sand filtration.

Laboratory Simulation of the Rapid Sand Filtration Process

Primary wastewater effluent from the heavily industrialized area of Niagara Falls in which the presence of mutagens had been demonstrated was used in these studies. (See section on Niagara Falls AWT Plant.) The effluent was applied to two sand columns (referred in the text as A and B) and samples of purified effluent were secured from the midpoint and bottom of each column. Samples were withdrawn from two depths so the efficiency of removal of mutagens could be related to column depth. The columns used represented sand collected from two different sand beds at the LGVSTP. A

detailed description regarding the operation of the sand columns is provided in the Appendix A. Organics from influent midpoint, and final effluents were recovered using polyurethane foam plugs and XAD resin as the concentration procedures. The crude organic mixtures were separated into chemical classes and assayed for mutagenicity using the Ames' Salmonella assay. Because of limitation on the sample volumes, assays could not be performed on unconcentrated wastewater and unfractionated wastewater concentrates. Limitations on the test material also restricted the number of tester strains that could be used in the Ames' assay. The strain selected were TA98, TA1538, and TA100. The separated fractions (except neutrals) were tested in the concentrations range representing 100 to 500 mℓ wastewater. Because of severe toxicity to Salmonella the separated neutrals (aliphatic, aromatic, and oxygenated fractions) were tested at concentrations representing 50 and 200 mℓ wastewater.

The studies with simulated sand filtration process were undertaken with two samples of Niagara Falls primary effluent collected approximately one month apart. The results of the studies are described below:

Rapid Sand Filtration of the Niagara Falls Primary Effluent Collected on February 20, 1979--

Severe mutagenicity was observed with many separated classes of chemicals of the influent and effluent samples collected during the first run. The mutagenic response was confined largely to Salmonella strains TA98 and TA1538. On further evaluation, a problem was noted with the primary data. This problem was detected because of poor correlation between duplicate plates in the Salmonella liquid suspension assay. It was determined that the poor duplicates were caused by an inadvertent cross-contamination of the Ames' tester strains during the liquid suspension assay procedure. An in-depth explanation of this problem is given in Appendix A. Only the results obtained with strains TA1538 and TA98 were susceptible to this problem. The results from the sand column A run were affected more severely by this problem making it impossible to assess if the increased number of colonies on plates was caused by mutagenesis or cross contamination of the tester strains. Therefore, the results obtained with strains TA98 and TA1538 for the first sand column run are not reported here. Salmonella strain TA100 which was not affected by this problem showed a mutagenic response in many separated fractions. Water soluble and weak acid fractions recovered from the primary effluent with XAD showed weak to moderate mutagenicity (ratios 1.5 to 2.5) in the absence of mammalian metabolic activation. The same fractions of the final effluent were also mutagenic. In addition, weak mutagenicity appeared in the ether insoluble fraction. Effluent from sand column B showed significantly more mutagenicity in these fractions than the effluent from column A.

The chemical classes recovered by polyurethane foam plugs from the primary effluent were nonmutagenic with Salmonella strain TA100. However, mutagens representing weak acid, ether insoluble, and water soluble classes were detected in the polyurethane foam concentrate of the final effluent. The mutagens gave rise to ratios (treated versus control) in the range of 1.5 to 2.1 in the absence of mammalian metabolic activation. These results suggest that low levels of mutagens may be introduced during the sand filtration process.

Rapid Sand Filtration of the Niagara Falls Primary Effluent Collected on March 19, 1979--

During the bioassay of the influent and effluent samples from the second collection at Niagara Falls, the procedure was modified to eliminate the problem of contamination of Salmonella strains TA98 and TA1538 with TA100. The modified procedure was used with all the samples except for the separated fractions of the primary effluent (influent for sand column) recovered by polyurethane foam plugs. Without knowing the quality of the influent wastewater, it was difficult to interpret the data obtained with polyurethane foam concentrates with respect to the quality changes during filtration through sand columns. Salmonella strain TA100, which was unaffected by the contamination problems, showed mutagenic activity in the presence of the strong acid fraction of the primary effluent recovered by polyurethane foam; the increase in reversion rate was fourfold over the control. The similar fraction from midpoint and final effluent of the sand columns A and B was nonmutagenic to strain TA100 suggesting that the mutagens of this class were efficiently removed during sand filtration. Mutagens of the ether insoluble class which were active in Salmonella strain TA1538 were, however, present in the final effluent from sand column B. The final effluent from column A on the other hand, contained aromatic neutral compounds with mutagenic activity. Due to the lack of mutagenicity data for TA98 and TA1538 for the influent sample, it is not possible to suggest the possible source of these compounds in the final effluent.

Separated fractions of the primary effluent recovered by XAD showed severe toxicity towards Salmonella strains TA98 and TA100. Toxicity was less severe with midpoint effluent and absent in the final effluent from the sand columns, indicating efficient removal of these toxicants during sand filtration. Mutagenic response was not observed in the tester strains with any separated fractions except for the ether insoluble fraction of the final effluent which exhibited mutagenicity in TA98 in the absence of metabolic activation.

While primary effluent showed no mutagenic response in strain TA1538, the water soluble and ether insoluble fractions of the midpoint sample were mutagenic (ratios 2.6-3.3) (Figure 35). Strong acid, weak acid, basic, and amphoteric fractions of the midpoint effluent were weakly mutagenic to strain TA1538 (ratio >1.5). The final effluent was generally free of mutagens. The appearance of mutagens in the midpoint sample could be caused by either differential removal of mutagens and toxicants by sand filtration, or by synthesis of new mutagenic compounds by the biological activity on the sand columns. It could not be determined which of the two mechanisms were occurring without determining what caused the lack of a mutagenic response in the primary effluent. However, since the primary effluent was highly toxic to strains TA98 and TA100, it was possible that TA1538 was also affected. This would suggest that removal of toxicants was at least partially responsible for the appearance of a mutagenic response in the midpoint sample. In any case, it appeared that filtration through the full seven feet of sand columns was effective in removing or destroying these mutagens.

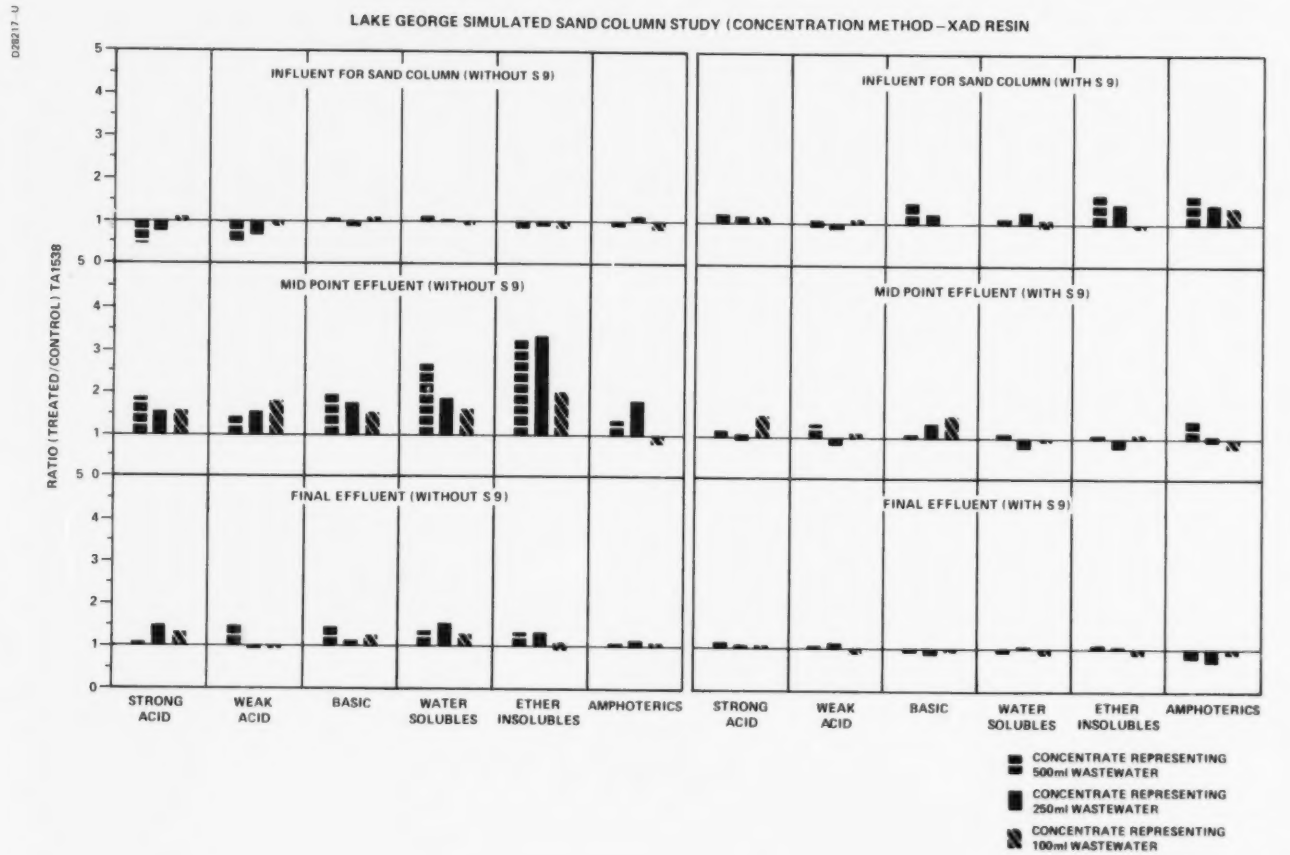

LAKE GEORGE SIMULATED SAND COLUMN STUDY (CONCENTRATION METHOD – XAD RESIN
D28217-U
RATIO (TREATED/CONTROL) TA1538
INFLUENT FOR SAND COLUMN (WITHOUT S 9)
INFLUENT FOR SAND COLUMN (WITH S 9)
MID POINT EFFLUENT (WITHOUT S 9)
MID POINT EFFLUENT (WITH S 9)
FINAL EFFLUENT (WITHOUT S 9)
FINAL EFFLUENT (WITH S 9)
STRONG ACID
WEAK ACID
BASIC
WATER SOLUBLES
ETHER INSOLUBLES
AMPHOTERICS
CONCENTRATE REPRESENTING 500ml WASTEWATER
CONCENTRATE REPRESENTING 250ml WASTEWATER
CONCENTRATE REPRESENTING 100ml WASTEWATER

Figure 35. Mutagenic activity of the major classes of compounds recovered from influent midpoint and final effluent of simulated sand column. Salmonella tester strain TA1538.

Fink (1974) has shown that filtration through the initial two feet of sand accomplished most of the carbon removal (nearly 50% to 60%) while subsequent travel distance gradually further reduced the level of organic carbon. The data on carbon removal is not in agreement with the removal of mutagenic chemicals by sand filtration. As shown earlier, mutagenic activity of wastewaters increased during the filtration through the initial few feet of the sand column but decreased during filtration through further depth of sand. The lack of agreement may reflect differences in the performance of sand in removing mutagens, and other chemicals which constitute the major portion of the organic carbon. It appeared more likely, however, that the difficulty in accurately measuring mutagenic activity in the presence of toxic substances present in wastewater, was responsible for these differences.

Conclusion

The data suggests that sand filtration was effective in removing a great deal of the organic toxicants from wastewater. In regard to mutagenic compounds, the data was less clear. The influent for the sand column did not show a significant level of mutagenic activity except in the strong acid fraction. After filtration of the wastewaters through half of the sand column, strong mutagenic activity appeared in the water soluble and ether insoluble fractions, and weak mutagenic activity appeared in weak acid, basic, and amphoteric fractions. Strong acid fraction, on the other hand, showed decreased mutagenicity after sand filtration. On continued contact with sand, mutagens of the amphoteric, weak acid, and basic classes were removed to nondetectable levels, but mutagens of ether insoluble, water soluble, and strong acid class were unaffected. The presence of several new classes of mutagens in the midpoint and final effluent, not detected in the influent, suggests synthesis of mutagens during the sand filtration process. More likely, however, is the possibility that removal of toxicants during filtration permitted expression of mutagens present in the influent.

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APPENDIX A

OPERATION OF SAND COLUMNS

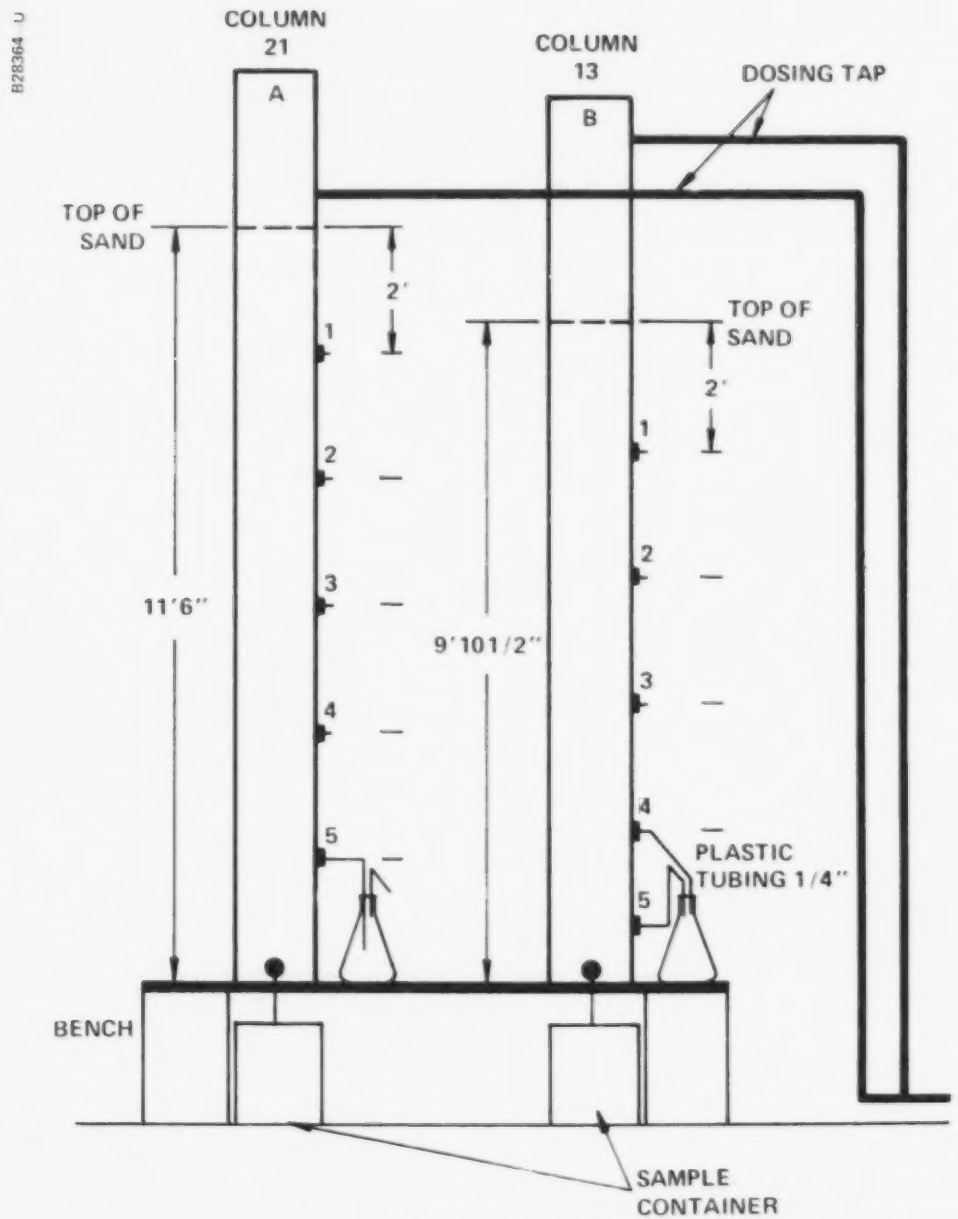
The sand columns were filled to the top with the appropriate sewage effluent. Since the top of the column was approximately 3 feet above the top of the sand, the approximate depth of loading was 3 feet or 0.9 m/day. The actual depth of loading was somewhat less than this since there were approximately 6 inches of glass beads on top of the sand at the top of the column. An actual measurement of the volume of the glass beads was not made, but it may be assumed that this reduced the depth of the application to about 0.8 m/day (2.6 feet/day). The general operation was to dose a bed once per day, providing that the liquid level had drained below the surface of the sand. Thus, the actual loading rate of the sand columns was greater than the normal loading rate in the sand beds.

Since the sand columns had not been used for at least one year prior to this study, the biological growth on the beds was reinstituted by passing treated (LGVSTP) effluent through the sand beds for a period of two weeks. During the first week, the beds were dosed twice a day because the infiltration rate was so great. As the biological system built up on the sand, the flow rate decreased and the dosing was performed only once per day. During this acclimatization period the applied sewage effluent was observed to have passed through the entire column prior to the next dosing. This was determined by the fact that the effluent dripping out of the bottom of the column had either ceased or diminished significantly.

In all cases the sewage effluent to be applied to the sand columns was kept under refrigeration from the time of sampling until the time of application. The sample containers were shaken prior to application to the sand column to resuspend any material which had settled out during the storage. The columns were operated at room temperature.

Primary effluent collected from the Niagara Falls Sewage Treatement Plant after flocculation, sedimentation, and acid mixing served as influent for the sand columns. A portion of the sample was deposited at the Syracuse Research Corporation for determination of quality prior to application to the sand columns. The remaining portion was transported to Rensselaer Polytechnic Institute where the column studies were conducted. The study was performed with two samples of the primary effluents collected on February 20, 1979 and on March 19, 1979.

During the acclimatization period when the LGVSTP effluent was applied to the columns, all of the side taps on the sand columns were collecting samples. However, shortly after applying the Niagara Falls effluent, the



Sand placed in column A was taken from bed N-13 at the LGVSTP.

Sand placed in column B was taken from bed S-7 at the LGVSTP.

side taps tended to plug and the only significant samples which could be secured were from the bottoms of the two columns. An effort was made to unplug at least one side tap in each column. Some samples were secured from one side tap in each column, but the volume was much less than that secured from the bottoms of the columns. Thus, a much greater volume of sample was secured from the column bottoms than the midpoint of each column.

Toward the end of the first study using the Niagara Falls effluent, the columns were observed to percolate the applied effluent at a much slower rate. Thus, after the first study was completed, the columns were allowed to rest for two weeks prior to the start of the second study. Throughout the study, all of the applied effluent passed into the column within any given 24 hour period. Thus, there was some aeration of the bed prior to the next application of sewage, and although the rate of application was higher than at Lake George, it was considered that the results should be similar to those obtained at the LSVSTP.

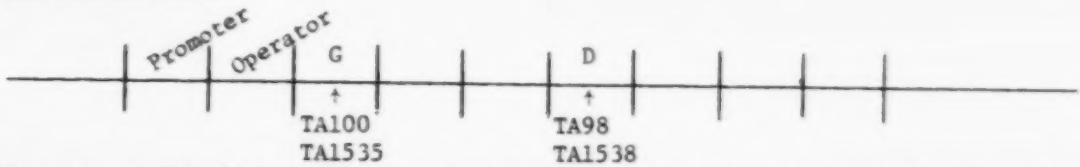
The column effluents were collected continuously. On a daily basis a portion of the total effluent was placed in a clean plastic container and frozen. At the end of the individual study the frozen samples were transported to the Syracuse Research Corporation for analysis.

DESCRIPTION OF THE DIFFICULTIES ENCOUNTERED WITH THE AMES' ASSAY DURING STUDIES WITH THE SAND COLUMNS

Background

The Salmonella strains used in the Ames' assay are blocked by mutations in different regions of the histidine operon. The genetic make-up of strains TA100 and TA1535 (His G mutants) and TA98 and TA1538 (His D) are shown below:

Histidine Operon



The mutants blocked by His D mutation may excrete an intermediate of the histidine biosynthesis pathway that can be utilized by His G mutants to produce histidine, i.e., His G mutants can then grow in the absence of exogenous histidine.

In simpler terms, if TA1538 or TA98 assay tubes become contaminated with TA100 (or TA1535), the TA100 auxotrophs presented with the histidine intermediate produced by TA1538 or TA98 will grow to form colonies in the histidine free media.

Source of Contamination

In the liquid suspension assay, appropriate tester strains, S-9 (when added) and the test mixture are allowed to incubate for one hour. There-

after, 3 ml soft agar containing traces of histidine and biotin are added, and the contents overlaid on minimal-glucose agar in a petri dish. Revertants are counted in the usual manner after 72 hours of incubation.

During the Ames' assay studies of the samples produced by sand columns, a single pipet was used for adding agar in all the assay tubes containing TA98, TA100, and TA1538 (in that order). This allowed the possibility for the pipet tip to pick up some TA100 cells and transfer them into TA1538 tubes. The TA100 cells presented with the histidine intermediate produced by TA1538 grew into colonies in TA1538 plates, thus confusing the results. The problem was also seen with TA98, although it was less intense. The TA100 contaminated pipet was left sitting in the molten top agar and was reused for the next series of tubes starting with TA98. This most likely led to the contamination of TA98 assay tubes with TA100 cells. Since the pipet was used to dispense sterilized agar, at the time nothing seemed wrong in using one pipet for agar for the entire experiment.

A routinely run sterility test performed on the top agar was always negative. However, this control was not designed to detect TA100 contamination, since the Salmonella strain will not grow on minimal-glucose agar in the absence of histidine or histidine precursors provided by TA98 or TA1538. This is how the contamination went undetected.

The problem was only noted with the liquid suspension assay. This is simple to explain, since in this form of the Ames' assay, agar is added to the assay tubes after the bacteria (in plate incorporation assay, bacteria are added last). The contamination probably arose during this set of experiments, because of a slight shift in pipeting technique which allowed the pipet tip to become contaminated. Assays performed prior to this experiment did not show signs of cross contamination of the tester strains on re-evaluation of the data. The problem was simply resolved by using separate pipets for transferring agar to the assay tubes of each strain.